Paclitaxel sensitivity related genes in gastric cancer

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Paclitaxel sensitivity related genes in gastric cancer

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Abstract

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Paclitaxel, a widely used anticancer drug, inhibits microtubule depolymerization during mitosis, which leads to cell death in various cancer types. Paclitaxel resistance has been widely studied, but the mechanism was confined to MDR1 and tubulin. Herein, we selected novel paclitaxel sensitivity related genes using a high-throughput method and validated their functions with respect to paclitaxel sensitivity. We constructed chemosensitivity and gene expression profiles for 78 human cancer cell lines using MTT-assays and oligonucleotide microarrays, respectively. Genes with differential expression between the resistant and sensitive groups were selected as paclitaxel sensitivity-related genes. Leave-one-out cross-validation (LOOCV) was performed for internal validation, and support vector machine (SVM) was used for *in vivo* chemosensitivity prediction with tumors derived from gastric cancer

cell lines. For functional evaluation of selected genes in gastric cancer cell lines, gene silencing was conducted with siRNA followed by cell viability assays, apoptosis assays, and cell cycle arrest analysis with paclitaxel treatment. We selected 115 genes that were related to paclitaxel sensitivity, for which LOOCV predicted an accuracy of 93%. The SVM demonstrated a prediction accuracy of 100% for the selected genes and demonstrated that these selected genes could be predictive markers for paclitaxel chemotherapy. After confirming the expression of the selected genes by quantitative and semi-quantitative RT-PCR in resistant and sensitive gastric cancer cell lines (NCI-N87 and MKN-45, respectively) and *in vivo* tumor tissues derived from NCI-N87 and MKN-45, functional studies were performed with LAMP2 and RARRES3 in NCI-N87 cell line. Using siRNAs, we observed that the expression of these genes was repressed. Subsequent cell viability assays, apoptosis assays, and cell cycle arrest analysis revealed that knockdown of these two genes restored paclitaxel sensitivity by increasing necrosis, late apoptosis, and cell cycle arrest at the G2/M phase. In conclusion, the selected genes may act as not only predictive markers for paclitaxel chemotherapy, but also as chemo-sensitizers for paclitaxel in chemo-naive gastric cancer patients.

Key Words: paclitaxel sensitivity, predictive marker, pharmacogenomics, gastric cancer

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I. INTRODUCTION

Chemotherapy is one of the most important modalities of cancer treatment and many investigators have tried to improve its efficacy and reduce its toxicity. However, there remains insufficient understanding of the targets of chemotherapeutic drugs, their mechanisms of action, and inter-individual variability in responses to these drugs. These defects are due in part to a lack of effective predictive markers for drug sensitivity. Clinical applications of the few predictive markers that have been identified thus far have been unsuccessful[1-6]. Previous studies have identified several marker gene sets *in vitro*, but these have not validated the selected genes in animal models or patients Therefore, in order to investigate the relationship between identified marker gene sets and the drugs, in vitro data needs to be applied in vivo to obtain clinical data[7-11]. Further, gene

knockdown (or over-expression) methods for functional validation of anticancer drug-related genes are required.

Gastric cancer ranks fourth among the most common cancers and is the second-leading cause of cancer-related deaths in worldwide[12], with an aggressive tumor doubling time of 40 to 80 days[13]. Currently, the major treatment modalities for gastric cancer are surgery and chemotherapy. Most patients who receive radical surgery also receive adjuvant chemotherapy.

The main anticancer drug used for advanced gastric cancer, 5-fluorouracil (5-FU), produces a single-agent response rate (RR) of only 12% and a median survival time (MST) of 7 months[14]. In the late 1990s, a randomized phase III trial conducted by the European Organization for Research and Treatment of Cancer announced that the MSTs were increased approximately 7 months with ELF (etoposide, leucovorin and bolus 5-FU), FUP (infusional 5-FU plus cisplatin) and FAMTX (5-FU, doxorubicin and methotrexate), respectively[15]. Another drug combination, irinotecan and cisplatin, produced a 48% RR and a 9 months MST[16]. The therapeutic impact of these results on survival has been modest at best. Therefore, new anticancer agents are needed to improve the survival of patients with metastatic gastric cancer.

Paclitaxel, a natural product-based anticancer drug derived from the bark of the Pacific Yew, *Taxus brevifolia*, inhibits microtubule depolymerization during mitosis followed by cell death[17] and is a more effective anticancer compound than older drugs[18], providing a broad spectrum of antitumor activity that includes lung, breast, and ovarian cancers[19,20]. Paclitaxel has demonstrated

substantial activity *in vitro* against MKN-28 and MKN-45 gastric carcinoma cell lines[21]. A phase II trial involving paclitaxel and gastric cancer demonstrated promising activity, with a RR of 20%[22]. In 1999, a combination of paclitaxel, 5-FU, and cisplatin was introduced to treat advanced gastric carcinoma with the tolerable toxicity[23]. Since then, paclitaxel has been used for gastric cancer both alone and in combination with other drugs with good response rates[24-28].

Despite these findings, the enormous clinical success of paclitaxel has been compromised by the emergence of drug resistance, which appears to be derived from several independent mechanisms[29]. The better characterized biological mechanisms mediating resistance to paclitaxel include the enhanced activity of the xenobiotics transporter MDR1/P-glycoprotein[30], and alterations in tubulin structure and tubulin expression levels[31,32]. Several studies, which have been contradictory, have searched for predictive markers of paclitaxel resistance in various cancers. Thus, there is a need to identify novel markers to predict paclitaxel sensitivity before clinical administration, especially in gastric cancer.

To identify genes that may predict paclitaxel sensitivity, we utilized genome-wide microarray technology. Microarray technology has been used to screen genes that are associated with chemosensitivity in cancer cell lines[2-5]. This technology has facilitated the analysis of genome-wide expression profiles that can efficiently generate information on a large scale in clinical or biological samples. Further, spotted oligonucleotide microarrays, with a 70-base length, provide high quality results, avoid clone validation, tracking, and maintenance, and minimize cross-hybridization[33-39].

To identify novel predictive markers that may predict paclitaxel sensitivity in gastric cancer, we used 78 human cancer cell lines for gene selection. We then performed predictive and functional validation studies with gastric cancer cell lines. Next, we selected genes using gene expression and chemosensitivity profiling of 78 human cancer cell lines *in vitro*. Internal validation of selected genes was used to confirm that our selections were reasonable. To confirm the prediction ability of the selected genes *in vivo*, we established xenograft tumors with gastric cancer cell lines. Consistent with our hypothesis, the genes correctly predicted in vivo chemosensitivity for paclitaxel. After knockdown of selected genes, functional validation by cell viability assays, apoptosis assays, and cell cycle arrest analysis demonstrated that changes in gene expression restored sensitivity to paclitaxel. These selected genes may act not only as predictive markers for paclitaxel chemotherapy, but also as chemo-sensitizers for paclitaxel in chemo-naive gastric cancer patients.

II. MATERIALS AND METHODS

1. Cell Lines and Culture

Among a total of 78 human cancer cell lines of various origins, 63 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA), and 15 cell lines were established from Korean cancer patients at the Cancer Metastasis Research Center (CMRC, Yonsei University College of Medicine, Seoul, Korea). The complete list of cell lines is presented in Table 1. Cells were cultured in proper media provided by the manufacturer supplemented with 10% FBS (Omega Scientific, Tarzana, CA, USA) and 100 U/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), and incubated at 37℃ in a 5% CO_2 humidified atmosphere. Media was replaced every 3 days.

2. RNA Preparation

Total RNA was extracted from each cell line using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Yonsei reference RNA (Cancer Metastasis Research Center, Seoul, Korea) was prepared by pooling equivalent amounts of total RNA from the following 11 human cancer cell lines: YCC-B1 (breast cancer), HCT-116 (colon cancer), SK-HEP-1 (hepatoma), A549 (lung cancer), HL-60 (acute promyelocyte leukemia),

Table 1. The origins and IC_{50}s of 78 human cancer cell lines

No.	Cell line	Origin	IC_{50} (uM)
1	HT-1376	Bladder cancer	0.046
2	RT4		0.003
3	IMR-32	Brain cancer	0.004
4	YCC-BRN		0.002
5	T98G		22.968
6	U87MG		13.007
7	MCF/ADR	Breast cancer	0.384
8	MCF-7		0.542
9	MDA-MB-231		0.064
10	MDA-MB-435		0.052
11	SK-BR-3		0.007
12	T47D		0.007
13	YCC-B1		8.015
14	YCC-B2		0.007
15	YCC-B3		9.467
16	YCC-B5		7.229
17	C33A	Cervix cancer	0.006
18	CaSki		0.006
19	Hela		0.006
20	SiHa		0.035
21	COLO 205	Colorectal cancer	0.006
22	DLD-1		0.051
23	HCT-116		0.058
24	HCT-15		0.053

25	HT 29		0.081
26	HT-1080	Fibrosarcoma	0.036
27	AGS	Gastric cancer	0.038
28	MKN-45		0.019
29	NCI-N87		8.284
30	SNU-1		1.652
31	SNU-484		0.011
32	YCC-1		8.893
33	YCC-2		9.137
34	YCC-3		10.586
35	YCC-6		8.280
36	YCC-7		15.282
37	YCC-10		7.978
38	YCC-11		6.931
39	YCC-16		8.016
40	HS746T		0.081
41	KATO III		0.002
42	MKN-1		0.026
43	MKN-28		0.602
44	MKN-74		0.182
45	SNU-5		0.039
46	SNU-16		0.001
47	SNU-216		2.050
48	SNU-638		0.004
49	SNU-668		0.051
50	HL-60	Hematologic cancer	0.048
51	Jurkat		0.004
52	Molt 4		0.054

53	Raji		1.708
54	Caki-2	Kidney cancer	11.965
55	HepG2	Liver cancer	2.077
56	Hep3B		7.166
57	SK-Hep-1		1.241
58	SNU-182		0.033
59	SNU-398		11.915
60	SNU-449		8.384
61	SNU-739		0.005
62	A549	Lung cancer	1.331
63	NCI-H1299		0.047
64	NCI-H460		0.042
65	NCI-H596		0.030
66	NCI-H647		0.905
67	NCI-H69		14.123
68	G361	Melanoma	31.122
69	SK-MEL-2		0.715
70	SK-MEL-24		8.607
71	YCC-O1	Ovary cancer	13.934
72	Capan-2	Pancreas cancer	6.342
73	YCC-YJH		0.005
74	LNCap	Prostate cancer	7.216
75	PC3		0.051
76	SCC-1438	Tongue cancer	7.058
77	SCC-15		7.056
78	SCC-25		0.333

MOLT-4 (acute lymphoblastic leukemia), HeLa (cervical cancer), Caki-2 (kidney cancer), T98G (glioblastoma), HT1080 (fibrosarcoma) and YCC-3 (gastric cancer)[40]. The quantity and quality of RNA was confirmed by an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and gel electrophoresis.

3. Chemosensitivity Assay

Growth inhibition was measured in 13 human gastric cancer cell lines with paclitaxel (Sigma, Saint Louis, MO, USA) using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, Saint Louis, MO, USA) assay. Each cell line was seeded into a 96-well microplate (BD Falcon, Franklin Lakes, NJ, USA) and incubated for 24 hours at 37℃ in a humidified 5% CO_2 atmosphere. Paclitaxel was serially diluted by culture media and various concentrations were added to each well. Following 72 hours of incubation, 50 μl (2 mg/ml) of MTT solution was added and incubated for an additional 4 hours. After centrifugation at 400 g for 10 minutes, the medium and MTT were removed from the wells and the remaining MTT-formazan crystals were dissolved by addition of 150 μl dimethyl sulfoxide (DMSO, Sigma, USA). Following 10 minutes of shaking incubation, the absorbance at 540 nm was measured with a multi-well ELISA automatic spectrometer (Behringwerke, Marburg, Germany). Results are expressed as percent cell survival, which was calculated using the following formula: % survival = [(mean absorbance of test

wells – standard absorbance) / (mean absorbance of control wells – standard absorbance)] x 100. Control wells were treated with medium alone (without the anticancer drug). Percent cell survival at varying drug concentrations was plotted to determine the growth inhibitory concentration. The drug concentration at which 50% of cancer cells survived (IC_{50}) was calculated using Calcusyn software (Biosoft, Cambridge, UK)[41]. Figure 1 shows IC_{50} calculation examples in the resistant cell line G361 and in the sensitive cell line KATO III. Because there were wide variations in the scale of data points for different drugs, the IC_{50} was transformed to a $\log_{10}$ scale.

4. Oligonucleotide Microarray

Oligonucleotide microarray analysis was performed using a human oligo chip (CMRC-GT, Seoul, Korea) containing 22,740 oligonucleotide probes of 70 bases based on a reference design. The test samples (RNA from each cell line) were labeled with Cy5 and individually co-hybridized with the Cy3-labeled reference RNA. One hundred micrograms of total RNA from each sample was mixed with oligo-dT primer (Genotech, Daejun, Korea) and incubated at 65°C for 10 minutes. After adding SuperScript II (Invitrogen, Carlsbad, CA, USA), 5X first strand buffer, 100 mM DTT, low-dT/dNTP mix, and Cy3- or Cy5-dUTP to the RNA/oligo-dT mixture, a reverse transcription process was performed at 42℃ for 2 hours. The remaining RNA was then hydrolyzed by incubation at 65℃ for 30 minutes in 0.1 N NaOH and the reaction was neutralized by

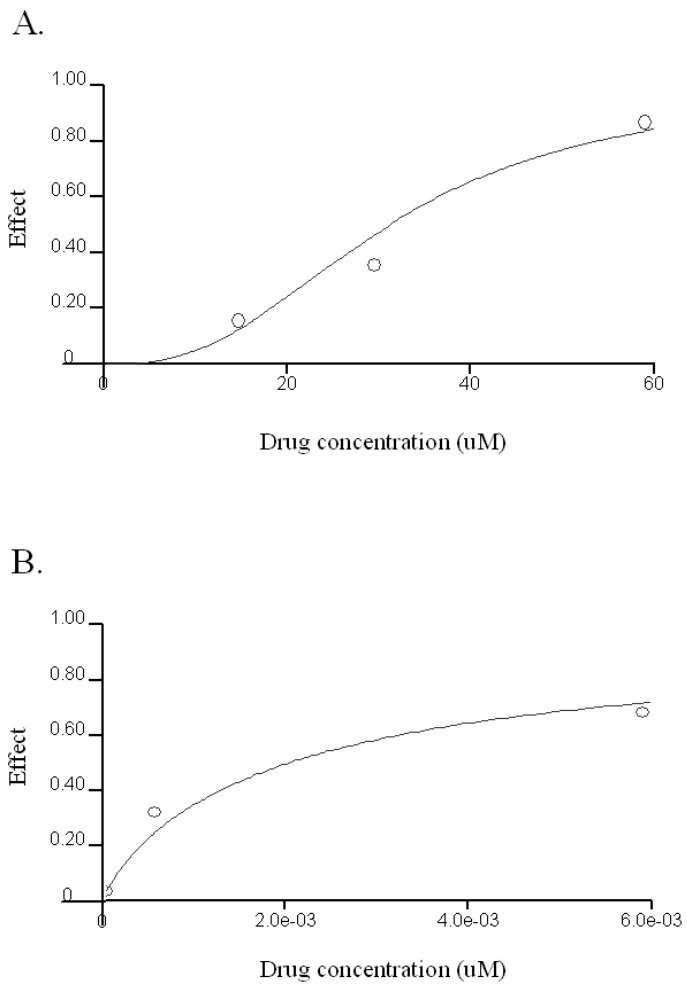

Figure 1. Dose-effect curve by calcusyn. A. An example of a resistant cell line, G361. The IC_{50} was calculated when the effect value on the y axis reached 0.5. B. An example of a sensitive cell line, KATO III.

addition of an equal volume of 0.1 N HCl. The Cy3- and Cy5-dUTP labeled probes were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). The purified probes were combined and mixed with Human Cot-1 DNA (Invitrogen, Carlsbad, CA, USA), yeast tRNA (Invitrogen, Carlsbad, CA, USA) and poly (A) RNA (Sigma, Saint Louis, MO, USA). The final probe was concentrated using a Microcon YM-30 column (Millipore, Bedford, MA, USA) and denatured at 100℃ for 2 minutes. Oligonucleotide microarrays were pre-hybridized in 5X sodium chloride/sodium citrate buffer (SSC), 0.1% sodium dodecyl sulfate (SDS), and 10 mg/ml bovine serum albumin (BSA) at 42℃ for 1 hour. Next, the probe was hybridized in 30% formamide, 5X SSC, and 0.1% SDS at 42℃ for 16 hours. Following hybridization, arrays were sequentially washed for 2 minutes in 2X SSC with 0.1% SDS, 1X SSC with 0.1% SDS, 0.2X SSC, and 0.05X SSC, and spin-dried at 500 g. Fluorescence was measured using a GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA) and the scanned images were processed using GenePix Pro 4.0 software (Axon Instruments, Foster City, CA, USA).

For further analysis, raw Cy5/Cy3 data were $\log_2$-transformed. Systemic errors were corrected by normalization using intensity dependent, within-print, tip normalization based on the Lowess function. After normalization, genes that were missing more than 20% of their values in all experiments were filtered. The values of repeated genes were adjusted by S-Plus 2000 software (Insightful, Seattle, WA, USA). Hierarchical clustering and its visualization were performed with the Cluster module of GeneSpring GX (Agilent Technologies, Santa Clara,

CA, USA).

5. Gene Selection

To select paclitaxel sensitivity-related genes, chemosensitivity and gene expression profiling were integrated. In the chemosensitivity data, cell lines with a $\log_{10}IC_{50}$ exceeding more than one standard deviation (SD) were defined as resistant to paclitaxel, whereas cell lines with a $\log_{10}IC_{50}$ under the one SD were defined as sensitive to paclitaxel. Cell lines with $\log_{10}IC_{50}$ within one SD were considered to be intermediates and were eliminated from analysis[5]. Using the Volcano plot of GeneSpring GX, genes differentially expressed over 1.5-fold with high significance ($p<0.01$) between resistant and sensitive cell lines were selected as paclitaxel sensitivity-related genes. To verify selected genes in resistant and sensitive cell lines, the Leave-one-out cross-validation[42] of GeneSpring GX was used. Annotation of the selected genes was performed using the Stanford Online Universal Resource for Clones and Expressed sequence tags (http://source.stanford.edu/cgi-bin/ source/ sourceSearch, SOURCE, Stanford, CA, USA)[43] and FatiGO (http://fatigo.bioinfo.cipf.es/, BABELOMICS, Valencia, Spain)[44].

6. Human Tumor Xenograft in Mice

For tumor cell implantation, 10^7 of the NCI-N87 resistant gastric cancer cell

line and the MKN-45 sensitive gastric cancer cell line were trypsinized and resuspended in 100 ul of PBS immediately prior to inoculation. Cells were injected subcutaneously into the right flank of 6-week-old Balb/c female athymic nu/nu mice. When tumors reached a volume of 200-300 mm^3, mice were randomly grouped into a control group and a treated group (five mice each). The day of the group assignments was defined as day 1. Tumor volume was measured every 2 days and the volumes were calculated using the equation $V= (Dxd^2)/2$, where V (mm^3) is the tumor volume, D is the longest diameter in mm, and d is the shortest diameter in mm[45]. Paclitaxel was administered i.p. at a dose of 15 mg/kg on days 1, 4, and 7[46-49]; the control group received 0.9% saline according to the same schedule. Tumor volume measurement continued until the 23rd day. At the time of termination, all of the mice were sacrificed and paclitaxel-naive control tumors were excised for gene expression profiling. Total RNA extraction from tumor tissues and oligonucleotide microarrays was conducted as described above. For the prediction of *in vivo* chemosensitivity with the selected genes, the support vector machine (SVM)[42] of GeneSpring GX was applied.

7. Quantitative and Semi-quantitative Reverse Transcriptase (RT)-PCR

To validate the expression of selected genes by RT-PCR, 4 ug of total RNA from each sample was mixed with oligo-dT primer and incubated at 65°C for 10 minutes. After adding SuperScript II, 5X first strand buffer, 100 mM DTT, and

10 mM dNTP mix to the RNA/oligo-dT mixture, a reverse transcription process was performed at 42℃ for 1.5 hours. The remaining RNA was hydrolyzed by incubation at 65℃ for 15 minutes in 0.1 N NaOH. The reaction was then neutralized by addition of an equal volume of 0.1 N HCl. The synthesized cDNA mixtures were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). For both semi-quantitative and quantitative RT-PCR, 200 ng of purified cDNA was used. Semi-quantitative RT-PCR (sqRT-PCR) was performed using Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and the amplified products were separated on ethidium bromide gels containing 1.2% agarose. The house-keeping gene GAPDH was used as a control for equal loading. Quantitative RT-PCR (qRT-PCR) was performed using QuantiTect SYBR Green PCR (QIAGEN, Valencia, CA, USA). Each reaction was run in duplicate on a Stratagene MX3005P (Stratagene, La Jolla, CA, USA). Expression values for each gene were determined using a standard curve constructed from Human Genomic DNA (Promega, Madison, WI, USA). The house-keeping gene ACTB was selected for normalization and the standard curve. Non-template-control wells without cDNA were included as negative controls. The primer sets for RT-PCR amplification are presented in Table 2. To compare gene expressions between the microarray and qRT-PCR data, an appropriate Pearson correlation coefficient was calculated.

8. Small Interfering RNA (siRNA) Transfection

Table 2. The primer sets for quantitative and semi-quantitative RT-PCR

No.	Gene symbol	Direction	Sequence (5'-3')
1	IL8	Left	CTTGTCATTGCCAGCTGTGT
		Right	GCCTTGTATTTAAAAATGCAGTCA
2	C3	Left	GACAAGGTCACCCTGGAAGA
		Right	ACTTGATGGGGCTGATGAAC
3	LAMP2	Left	TGCCTTGGCAGGAGTACTTA
		Right	TCTCAAAATGCTGGGATTGAT
4	ENC1	Left	TATTTTGTGGGCTGGTTGCT
		Right	TGAACATGAACATTTTCCTTCAA
5	RARRES3	Left	TGAAGCAGCCACAAAATCCT
		Right	GGGCAGATGGCTGTTTATTG
6	ARHGAP18	Left	TGTGACAAGAGATGAGCCTCTG
		Right	ATCATGAACACCGTTTGTGC
7	DDX58	Left	GCCATTACACTGTGCTTGGA
		Right	CCTCCACCACAAAACTTTCAA
8	SERPINA5	Left	TGGGGCTACTGTTTCAGTCC
		Right	AAAGGATGAATGGCACTTGA
9	P2RX5	Left	GGAGTCCTTGAGCACCTCAG
		Right	GGCAGGAAGGTGGTGTCTT
10	SRPK1	Left	GGAAATGCTTCTCCACCAAA
		Right	GTGAGATCGTGGCAATTTGA
11	SOX4	Left	CACGGTCAAACTGAAATGGA
		Right	ACTGACTGCCCCTGTACCAC
12	GAPDH	Left	CCATGGAGAAGGCTGGGG
		Right	CAAAGTTGTCATGGATGACC

13	ACTB	Left	GGGAATTCAAAACTGGAACGGTGA
		Right	GGAAGCTTATCAAAGTCCTCGGCC

Twenty-four hours prior to transfection, various numbers of cells were suspended in growth medium without antibiotics and seeded in a plate. The cell density was 30-50% confluence at the time of transfection. Transfection of siRNAs was carried out with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). Stealth siRNAs (Invitrogen, Carlsbad, CA, USA) targeting LAMP2 or RARRES3 were mixed with Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA, USA). This mixture was then mixed with an equal volume of Lipofectamine RNAiMAX in Opti-MEM I Reduced Serum Medium. After 20 minutes of incubation, the final mixtures were added to each well of the plate for a final siRNA concentration of 2 nM. After a minimum of 48 hours of incubation, cells were harvested and used for further analysis. The sequences of all siRNAs are listed in Table 3. A Stealth RNAi Negative Control Duplex (Invitrogen, Carlsbad, CA, USA) was used for non-targeting siRNAs (NT siRNA) as a negative control.

9. Immunoblot Analysis

A total of $2x10^5$ cells of the NCI-N87 cell line were plated into on each well of a 6-well plate. After 24 hours, Lipofectamine RNAiMAX (control), NT siRNA,

Table 3. The sequences of siRNAs for LAMP2 and RARRES3

Gene symbol	Target site	Direction	Sequence (5'-3')
LAMP2	Site 1	Left	AUUAAGUUCCAAUGCAUAAGACCGC
		Right	GCGGUCUUAUGCAUUGGAACUUAAU
	Site 2	Left	UUUGUAGUUUCAUAGGGUACUGUGA
		Right	UCACAGUACGUCAUGAAACUACAAA
	Site 3	Left	AAAGCUUGUACAAGAACAUCCCAGU
		Right	ACUGGGAUGUUCUUGUACAAGCUUU
RARRES3	Site 1	Left	AUAUACAGGGCCCAGUGCUCAUAGC
		Right	GCUAUGAGCACUGGGCCCUGUAUAU
	Site 2	Left	UGACCAACCAUCUCCUUCGCAGAAC
		Right	GUUCUGCGAAGGAGAUGGUUGGUCA
	Site 3	Left	AAAGAGCAUCCAGCAACAACCAGGA
		Right	UCCUGGUUGUUGCUGGAUGCUCUUU

and siRNA were treated as mentioned above for 48 hours. To confirm the inhibition efficiency of siRNA, siRNA treated cells were incubated for an additional 72, 96, 120 hours. The media was discarded and the cells were washed twice with PBS. Then, 60 ul of lysis buffer (50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, 10 ug/ml aprotinin, 10 ug/ml leupeptin, 1 mM PMSF) was added. Cells were detached by scrapping, incubated on ice for 30 min, and separated by centrifugation for immunoblotting. Whole cell lysates were separated by SDS-PAGE using 9.4%

polyacrylamide gels. Proteins were transferred onto PVDF membranes and analyzed by immunoblotting using antibodies against LAMP2, 1/1000, and RARRES3, 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary sheep anti-mouse IgG-HRP (GE healthcare, Buckinghamshire, UK) and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at 1/2000 and 1/1000 dilutions, respectively. The membrane was developed by ECL Western blotting reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). Anti-GAPDH (Abcam, Cambridge, UK) was used to control for equal loading.

10. Cell Viability Assay

Twenty-four hours before transfection, 1.5 x 10^4 cells of the NCI-N87 cell line were plated on each well of a 48-well plate. Cells were treated with Lipofectamine RNAiMAX (control), NT siRNA, and siRNA as detailed above for 48 hours. After washing each well with PBS, paclitaxel was serially diluted in culture media and various concentrations were added to each well (0-6 uM). Following 72 hours of incubation, the MTT-assay was performed as described in the chemosensitivity assay section.

11. Apoptosis Assay

Apoptosis in cells was evaluated by double staining with fluorescein-

isothiocyanate (FITC)–labeled annexinV (BioBud, Seoul Korea) 7-AAD (Calbiochem, La Jolla, CA, USA). A total of $2x10^5$ NCI-N87 cells were plated on each well of a 6-well plate. After 24 hours, Lipofectamine RNAiMAX (control), NT siRNA, and siRNA were added as mentioned above for 48 hours. Following treatment, the media was discarded and each well was washed with PBS twice. Three milliliters of growth medium with 10% FBS or 6nM of paclitaxel in the same medium were then added to each well for 48 hours. The cells were trypsinized and washed twice with PBS, suspended with 500 ul of 1x binding buffer (BioBud, Seoul Korea), followed by the addition of 1.25 ul of annexin V-FITC. After incubation for 15 minutes at room temperature in dark conditions, cells were washed with PBS and resuspended in 500 ul of 1x binding buffer, followed by the addition of 1 ul of 1 mg/ml 7-AAD. After an additional 72 hours of incubation at room temperature in the dark, cells were washed with PBS and resuspended in 500 ul of PBS. The final concentration of cells was approximately 10^6 cells/ml. As soon as possible, stained cells were analyzed by flow cytometry and the population was separated into four groups: live cells were shown only at the lower left panel, apoptotic cells were shown at the lower right panel, necrotic death was shown at the upper left panel, and late apoptosis was shown at the upper right panel.

12. Cell Cycle Analysis

Cell cycle analysis was performed by staining with propidium iodide (PI) (BD

Biosciences, San Jose, CA, USA). A total of $2.5x10^5$ NCI-N87 cells were plated into each well of a 6-well plate. Following plating, siRNA and paclitaxel treatment were conducted in the same manner as in the apoptosis assay except that the duration of paclitaxel incubation was 24 hours. The washed cells were resuspended in 1 ml of PI solution, and incubated for 15 minutes at RT in dark conditions. Following washing with PBS, cells were resuspended in 500 ul of PBS and analyzed by flow cytometry. The cell population of the G2/M phase was counted for comparison.

13. Statistical Methods

Statistical significance of the data was evaluated by calculating the student t-test for comparison between any two groups (triplicated data) and the Kolmogorov-Smirnov test[50] for comparison between any two whole chemosensitivity data sets.

III. RESULTS

1. Chemosensitivity Profiling of 78 Human Cancer Cell Lines

Paclitaxel was tested for growth inhibition in 78 human cancer cell lines. Twenty six cell lines with a $\log_{10}IC_{50}$ over 1 SD were defined as the resistant group and the 17 cell lines with a $\log_{10}IC_{50}$ under the 1 SD were defined as the sensitive group (Figure 2.). Whether a cell lines was resistant or sensitive groups did not appear to be related to its origin (resistant group: 2 brain, 3 breast, 9 gastric, 2 head and neck, 1 kidney, 3 liver, 1 lung, 2 melanoma, 1 ovary, 1 pancreas, and 1 prostate; sensitive group: 1 bladder, 1 blood, 2 brain, 2 breast, 3 cervix, 1 colorectal, 5 gastric, 1 liver, and 1 pancreas). All cell lines in the resistant group had higher IC_{50}s and all cell lines in the sensitive group had lower IC_{50}s than the maximum concentration of paclitaxel, which was 4.27 uM. The IC_{50}s of all 78 cell lines are listed in Table 1.

2. Gene Expression Profiling of 78 Human Cancer Cell Lines

To investigate the genetic characteristics of each cell line, we performed gene expression profiling for 78 untreated cancer cell lines using oligonucleotide microarrays. A total of 19,494 genes that satisfied the non-missing proportion (NMP) of 80% among the 78 cell lines were selected for further analysis. When unsupervised hierarchical clustering was conducted using the $\log_2$ (Red/Green)

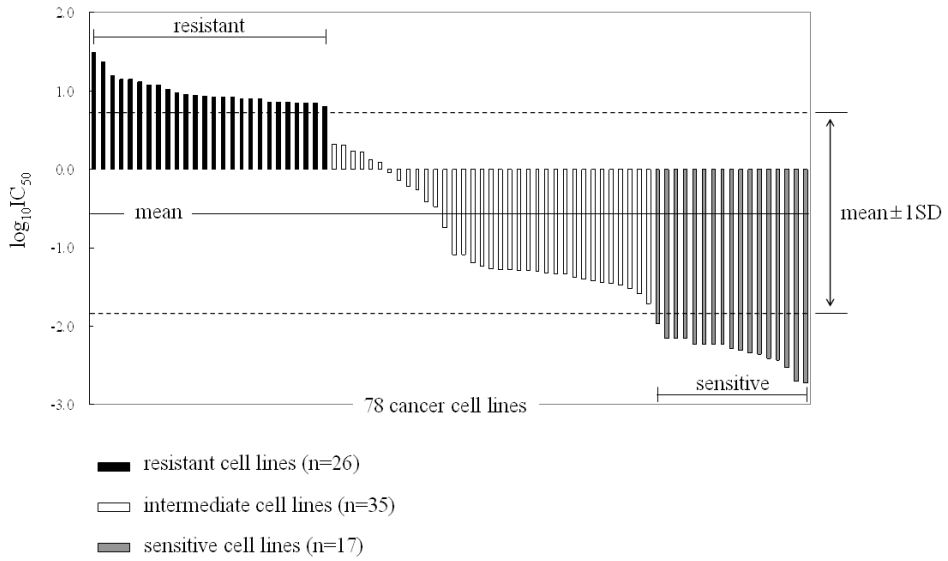

Figure 2. Chemosensitivity profiling of 78 human cancer cell lines. Mean ± 1 SD of $\log_{10}IC_{50}$ of the cell lines was applied to divide the cell lines into resistant and sensitive groups. Twenty-six cell lines, represented by black bars, which had a $\log_{10}IC_{50}$ 1 SD over the mean were defined as the resistant group and 17 cell lines, represented by grey bars, which had a $\log_{10}IC_{50}$ 1 SD less than the mean were defined as the sensitive group. Intermediates, represented by white bars, were excluded from further analyses.

ratio there were several clusters of cell lines in which the cell lines clustered together due to the similarity of their origins (Figure 3.).

3. Selection of Paclitaxel Sensitivity Genes

First, we performed unsupervised hierarchical clustering with 43 resistant and sensitive cell lines. The cell lines were not clustered according to paclitaxel sensitivity, but rather according to their origins. Thus, we attempted to select genes which were specifically related to paclitaxel sensitivity. Using the Volcano plot of GeneSpring GX, we selected 115 genes that were differentially expressed between the resistant and sensitive groups with a difference of at least 1.5-fold, indicating statistical significance ($p<0.01$) (Figure 4.). The 115 selected genes are listed in Table 4. The 115 genes that were selected comprised 48 up-regulated genes and 67 down-regulated genes in the resistant group. Supervised hierarchical clustering of the 115 genes demonstrated good correlation with the resistant and sensitive cell lines (Figure 5.). To statistically confirm this classification result, Leave-one-out cross-validation was performed with the resistant and sensitive cell lines as an internal validation of the 115 genes. The results demonstrated a prediction accuracy of 93%, indicating that it was reasonable to associate the genes with paclitaxel sensitivity (Table 5).

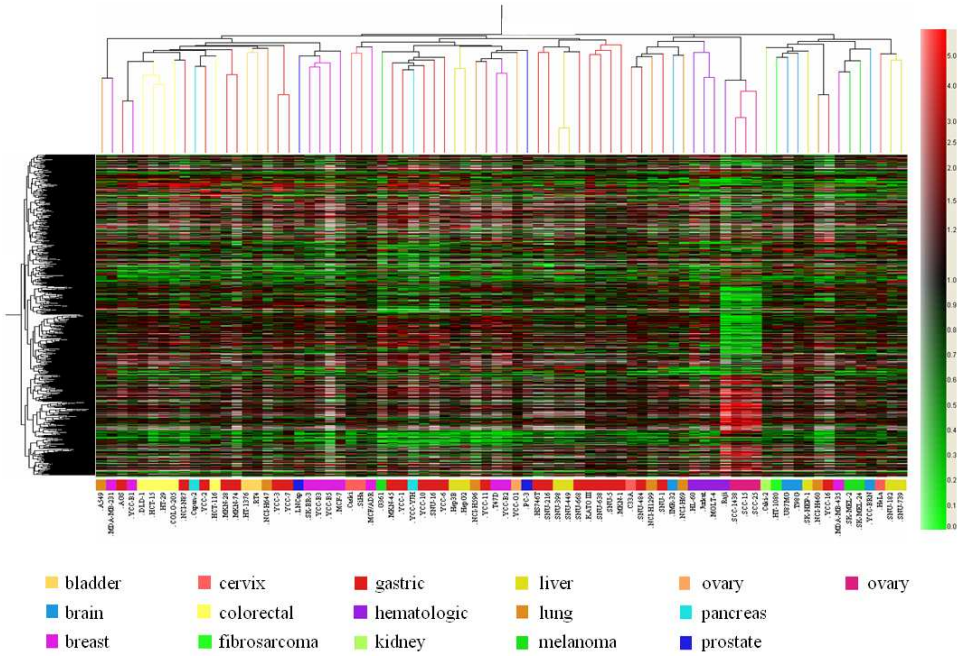

Figure 3. Gene expression profiling of 78 human cancer cell lines. Unsupervised hierarchical clustering of 78 cell lines and 19,494 genes (NMP 80%) showed that cell lines which had similar origins tended to be clustered together. The color bars under the treeview represented the origins of the cell lines. The scale bar on the right side of treeview indicates the expression values; red denotes high expression and green denotes low expression.

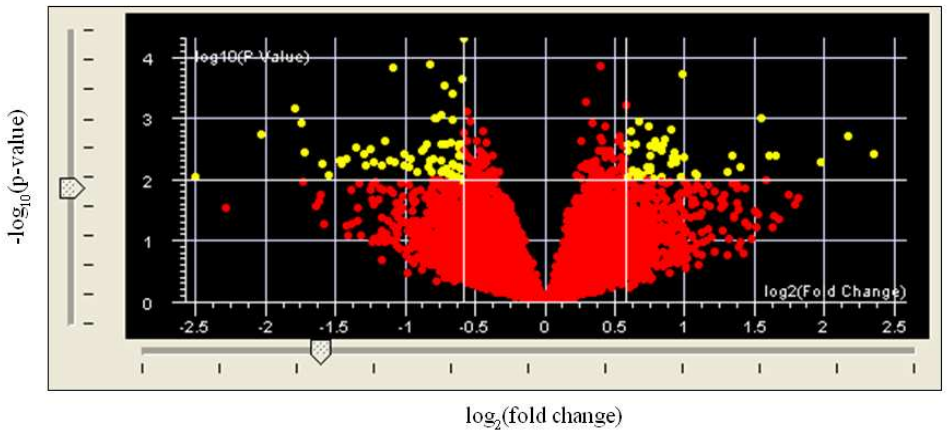

Figure 4. Selection of paclitaxel sensitivity-related genes by the Volcano plot of GeneSpring GX. With the criteria of fold change (>1.5) and p-value (<0.01), 115 genes, represented by yellow spots, were differentially expressed between the resistant and sensitive groups and were selected as paclitaxel sensitivity-related genes.

Table 4. Informations and expression values in each resistant and sensitive group of selected 115 genes

Status	Gene symbol	Expression value (log2R/G)	
		Mean of resistant group	Mean of sensitive group
Up-regulated in resistant group	IL8	-0.73	-2.99
	C3	0.33	-1.77
	EST	0.58	-0.10
	IFNA1	0.67	0.08
	PECR	0.59	-0.13
	SERPINA5	0.87	0.07
	SAT	-0.19	-1.57
	EST	0.45	-0.24
	EST	0.87	0.20
	BTD	0.93	0.19
	SCARB2	0.60	-0.21
	TNS3	-0.35	-1.72
	ARHGAP18	-0.47	-1.87
	TSPAN16	1.34	0.62
	EST	0.10	-0.82
	DOCK5	0.47	-0.54
	PDK4	-1.16	-1.94
	C1orf51	1.49	-0.45
	EST	1.05	0.33
	EST	0.55	-0.06
	EST	0.41	-0.24
	LAMP2	0.84	-0.08

	AFF4	0.81	-0.01
	C18orf25	0.44	-0.18
	GPR98	1.08	0.54
	GRIN2A	0.76	0.05
	RARRES3	0.49	-0.85
	DDX58	0.63	-0.18
	GLS	0.43	-0.43
	SPATA1	0.85	0.40
	EST	0.70	0.21
	FRK	1.34	-0.16
	EST	0.76	0.18
	ENC1	-0.32	-1.65
Up-regulated in	EST	0.64	-0.15
resistant group	PADI4	0.56	0.01
	EST	0.65	0.09
	NGB	0.52	0.07
	EST	1.19	0.59
	CCL19	0.89	0.40
	PER3	0.65	-0.32
	SFTPD	1.04	0.48
	C2orf39	0.69	0.02
	EST	1.34	0.67
	EST	1.03	0.42
	CDKN3	0.17	-0.46
	DAG1	0.80	0.18
	DLEC1	0.74	0.08

	GNL1	-0.19	0.42
	C9orf37	-0.17	0.43
	ZNF22	-1.80	-0.51
	EST	-1.91	-0.03
	FLJ12949	-0.44	0.12
	EST	-0.18	0.39
	RBMX	-1.06	-0.52
	CCNB1IP1	-0.79	-0.05
	EST	-0.56	0.53
	CROCC	-0.12	0.51
	EST	-1.06	-0.14
	EST	-0.37	0.16
	UPF3B	-0.38	0.27
Down-regulated in resistant group	RP11-82K18.3	-0.58	0.18
	C11orf57	-0.13	0.47
	EST	-0.40	0.20
	SMARCC1	-0.75	0.12
	TOP3A	-0.28	0.69
	SRPK1	-0.65	0.06
	MCM2	-0.68	0.12
	RPP21	0.00	0.58
	POLRMT	-0.46	0.14
	GCN5L2	-0.45	0.39
	CCDC25	-0.18	0.45
	RPL39	-0.21	0.14
	EST	-0.55	0.21
	ZNF44	-0.76	-0.17

	ZNF184	0.12	0.82
	EST	-0.49	0.60
	U2AF1L4	-0.01	0.53
	EST	-0.69	-0.08
	PSRC2	-0.98	-0.36
	EST	-1.38	-0.74
	EST	-0.85	-0.05
	MAP6D1	-0.17	0.87
	BLMH	-0.71	-0.17
	PGAP1	0.06	1.02
	KIT	-1.08	0.00
	PKIA	-1.19	0.44
	EST	-0.23	0.40
Down-regulated in resistant group	SOX4	-1.13	-0.02
	AYTL2	-0.69	-0.01
	PTPRCAP	-1.28	-0.75
	EST	-0.82	-0.28
	SIT1	-1.84	-0.84
	CD48	-3.24	-1.99
	DLEU8	-0.58	0.22
	P2RX5	-1.02	0.28
	SEPT6	-1.48	-0.63
	RORB	-1.67	-0.25
	USP44	-2.79	-1.26
	ZNRF1	-0.22	0.30
	RPL34	-0.39	0.23
	LRP16	-0.39	0.33
	RASIP1	-1.01	0.24

Down-regulated	PLCG2	-0.65	-0.07
in resistant group	EST	-3.20	-1.90
	TLX1	0.03	0.75
	MARCH6	-0.48	0.11
	SERPINF1	-0.06	1.07
	PNMA2	-1.12	-0.17
	PDPN	-0.89	-0.04
	CCND2	-3.79	-2.07
	CRYGD	-1.88	-0.58
	XAGE1	-0.72	0.28
	XAGE1	0.01	1.00
	XAGE3	-2.61	0.11

4. *In vivo* Prediction of Paclitaxel Sensitivity

To test whether the selected 115 genes were also reliable *in vivo*, especially in gastric cancer, we selected the paclitaxel resistant gastric cancer cell line, NCI-N87, and the sensitive gastric cancer cell line, MKN-45. The MKN-45 cell line was not included in the sensitive cell lines, however, because it was the most sensitive cell line among the intermediate group. Because there were no tumorigenic gastric cancer cell lines in the sensitive group, we selected MKN-45 as a sensitive cell line. Before introducing the MKN-45 cell line, we predicted the chemosensitivity of MKN-45 using SVM with the selected 115 genes. As a result, MKN-45 was predicted as a cell line sensitive to paclitaxel.

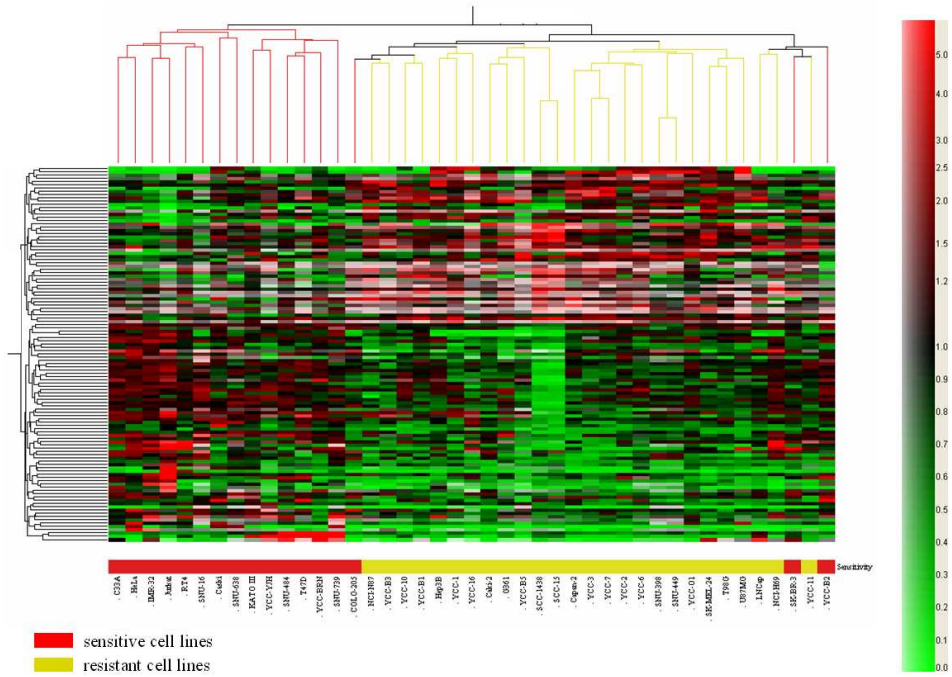

Figure 5. Supervised hierarchical clustering of the selected 115 genes with resistant and sensitive cell lines. Supervised hierarchical clustering showed that the 115 genes were well classified into resistant and sensitive cell line groups. The genes were composed of 48 up-regulated genes and 67 down-regulated genes in resistant group. The color bars under the treeview indicate the sensitivity to paclitaxel. The scale bar on the right side of treeview is to the same as that in Figure 3.

Table 5. The result of Leave-one-out cross-validation of 43 resistant and sensitive cell lines with selected 115 genes

Cell line	True value	Prediction	Resistant margin	Sensitive margin
Caki-2	Resistant	Resistant	1.572	-1.572
Capan-2	Resistant	Resistant	1.232	-1.232
G361	Resistant	Resistant	1.064	-1.064
Hep3B	Resistant	Resistant	1.131	-1.131
LNCap	Resistant	Sensitive	-0.0496	0.0496
NCI-H69	Resistant	Resistant	0.242	-0.242
NCI-N87	Resistant	Resistant	0.734	-0.734
SCC-1438	Resistant	Resistant	1.576	-1.576
SCC-15	Resistant	Resistant	1.509	-1.509
SK-MEL-24	Resistant	Resistant	1.68	-1.68
SNU-398	Resistant	Resistant	1.416	-1.416
SNU-449	Resistant	Resistant	1.564	-1.564
T98G	Resistant	Resistant	1.291	-1.291
U87MG	Resistant	Resistant	1.247	-1.247
YCC-1	Resistant	Resistant	0.929	-0.929
YCC-10	Resistant	Resistant	1.036	-1.036
YCC-11	Resistant	Resistant	0.886	-0.886
YCC-16	Resistant	Resistant	0.34	-0.34
YCC-2	Resistant	Resistant	0.507	-0.507
YCC-3	Resistant	Resistant	1.464	-1.464
YCC-6	Resistant	Resistant	1.11	-1.11
YCC-7	Resistant	Resistant	1.776	-1.776
YCC-B1	Resistant	Resistant	0.376	-0.376

YCC-B3	Resistant	Resistant	0.739	-0.739
YCC-B5	Resistant	Resistant	1.436	-1.436
YCC-O1	Resistant	Resistant	0.342	-0.342
C33A	Sensitive	Sensitive	-0.918	0.918
Caski	Sensitive	Resistant	0.238	-0.238
COLO-205	Sensitive	Resistant	0.733	-0.733
HeLa	Sensitive	Sensitive	-1.078	1.078
IMR-32	Sensitive	Sensitive	-1.63	1.63
Jurkat	Sensitive	Sensitive	-2.37	2.37
KATO III	Sensitive	Sensitive	-1.34	1.34
RT4	Sensitive	Sensitive	-0.756	0.756
SK-BR-3	Sensitive	Sensitive	-0.323	0.323
SNU-16	Sensitive	Sensitive	-1.344	1.344
SNU-484	Sensitive	Sensitive	-1.483	1.483
SNU-638	Sensitive	Sensitive	-0.972	0.972
SNU-739	Sensitive	Sensitive	-1.162	1.162
T47D	Sensitive	Sensitive	-0.662	0.662
YCC-B2	Sensitive	Sensitive	-0.805	0.805
YCC-BRN	Sensitive	Sensitive	-0.0128	0.0128
YCC-YJH	Sensitive	Sensitive	-0.647	0.647

Xenograft tumors were established with these cell lines in the subcutaneous tissue of 5 Balb/c nu/nu mice with each cell line. While paclitaxel-treated tumors derived from NCI-N87 demonstrated no significant differences from

saline treated tumors (Figure 6A.), paclitaxel-treated MKN-45 tumors showed significant inhibition compared with the saline treated group (Figure 6B.). Thus, we defined the NCI-N87 tumors as resistant tumors and the MKN-45 tumors as sensitive tumors. There were no body weight changes during the experimental dates (Figure 7.). To obtain the gene expression profiles of tumor tissues, paclitaxel naive control tumors were excised and an oligonucleotide microarray was performed using total RNA samples from the tissues as mentioned in material and methods. Each triplicate of gene expression showed that the resistant and sensitive tumors had genomic differences that were highly similar within each group (Figure 8.). When we performed supervised hierarchical clustering using the 115 genes with resistant/sensitive cell lines and tumor tissues, we confirmed that the resistant tumors were clustered with the resistant cell lines and that the sensitive tumors clustered with the sensitive cell lines (Figure 9.). To statistically confirm this classification result, SVM was performed with 115 genes based on the resistant and sensitive cell lines. The result showed 100% prediction accuracy (Table 6). In other words, these genes correctly predicted in vivo tumor chemosensitivity, indicating that they may be able to serve as predictive markers for paclitaxel chemotherapy.

5. Gene Annotation of Selected Genes

The 115 selected genes comprised of 87 known genes and 28 ESTs. When we performed functional annotation of the 115 genes using the criteria of over 10%

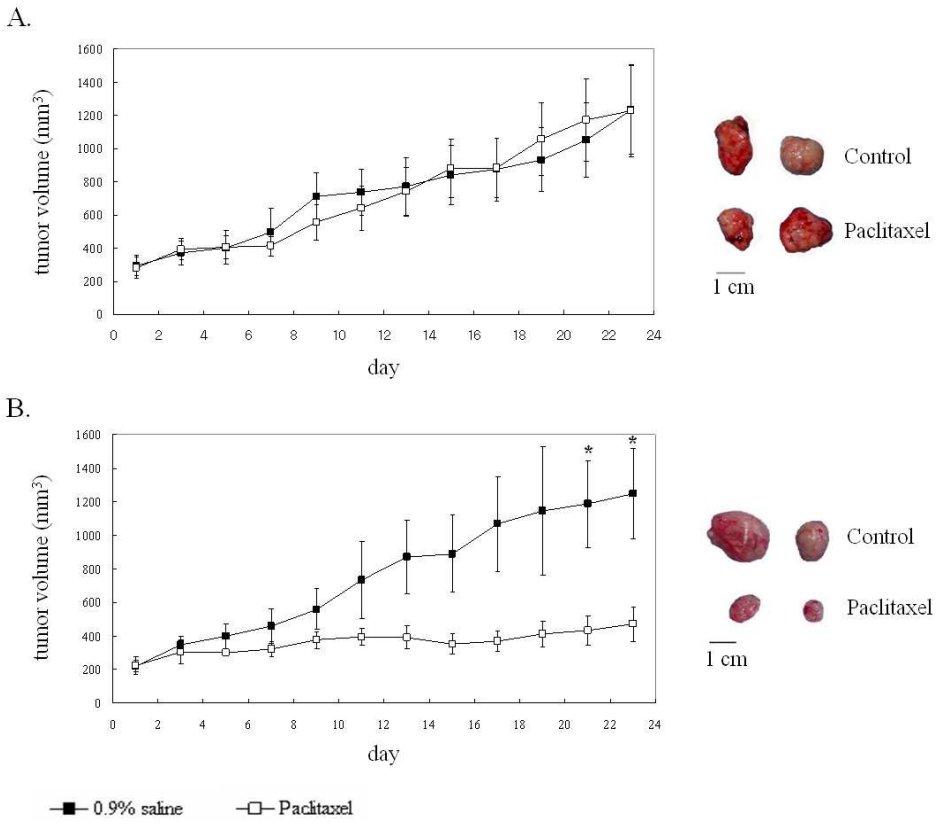

Figure 6. Tumor growth curve of NCI-N87 and MKN-45 derived tumors. When tumor volumes reached 200-300 mm^3 (day 1), 15 mg/kg of paclitaxel and 0.9% saline were administered i.p. on days 1, 4, and 7. A. Paclitaxel-treated NCI-N87 derived tumors (n=5) showed no significant difference from saline treated control tumors (n=5) ($p>0.05$). B. Paclitaxel treated MKN-45 derived tumors (n=4) showed inhibited growth and smaller tumor volumes compared with the saline-treated control group (n=4). * Differences in tumor volume between the control and the treated mice were statistically significant on the 21st and 23rd days ($P<0.05$, Student's t-test). Bars, SE.

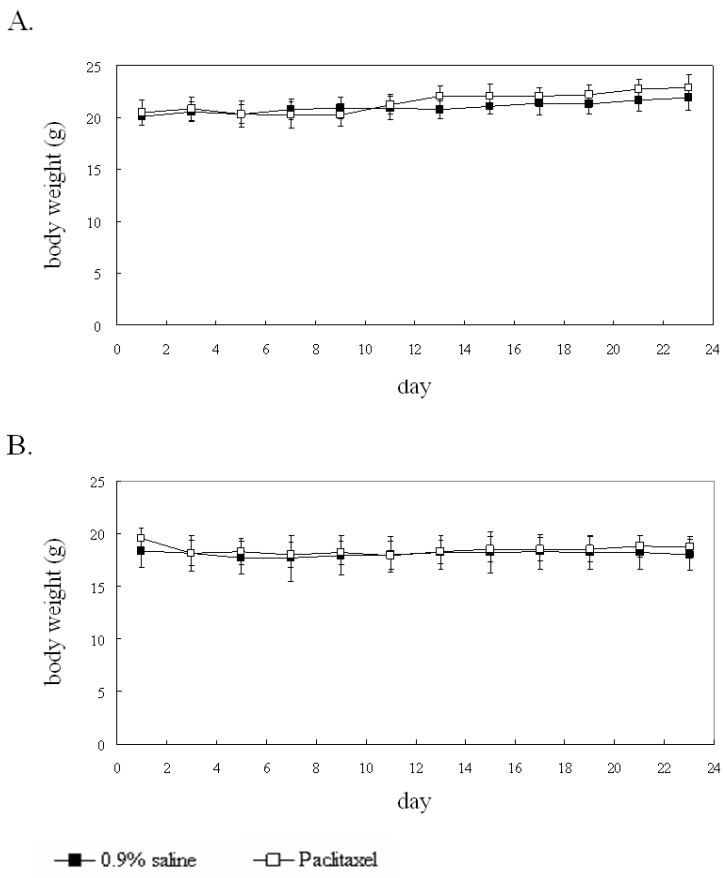

Figure 7. Body weight change during the experiment. A. Body weight of NCI-N87 derived tumors. B. Body weight of MKN-45 derived tumors. There was no change between the paclitaxel treated and the control groups. Bars, SD.

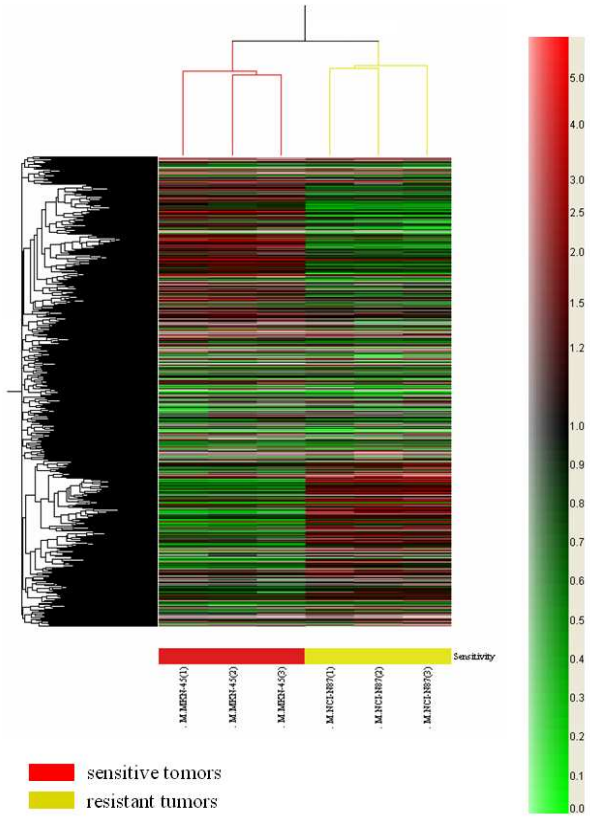

Figure 8. Unsupervised hierarchical clustering of three resistant tumors (yellow) and three sensitive tumors (red) with 15,846 genes satisfied NMP 80%. Gene expression measurements in triplicate showed that the resistant and sensitive tumors had genomic differences, with a high degree of similarity within each group.

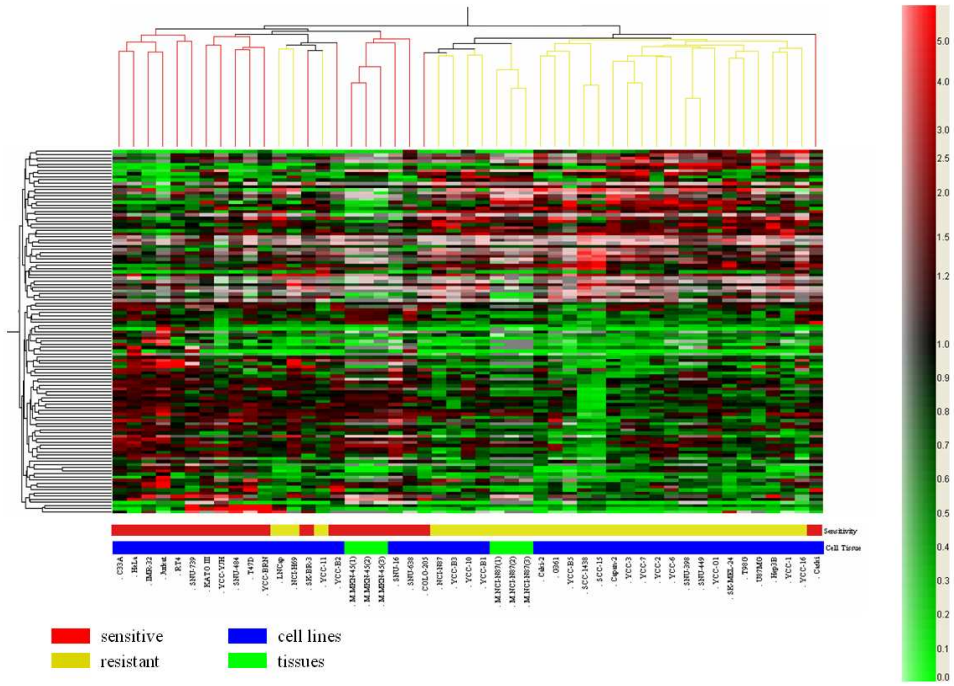

Figure 9. Supervised hierarchical clustering of 115 genes with resistant/sensitive (yellow/red) cell lines (blue) and tumor tissues (green). Resistant tumors clustered with resistant cell lines and sensitive tumors clustered with sensitive cell lines.

Table 6. The result of support vector machine of 6 resistant and sensitive tumor tissues derived from gastric cancer cell lines with selected 115 genes

Sample	True value	Prediction	Resistant margin	Sensitive margin
MKN-45 (1)	Resistant	Resistant	1.464	-1.464
MKN-45 (2)	Resistant	Resistant	1.572	-1.572
MKN-45 (3)	Sensitive	Sensitive	-1.078	1.078
NCI-N87 (1)	Resistant	Resistant	1.291	-1.291
NCI-N87 (2)	Resistant	Resistant	1.776	-1.776
NCI-N87 (3)	Resistant	Resistant	0.507	-0.507

enrichment with statistical significance ($p<0.05$), these genes were found to be involved in cellular metabolic process, the regulation of biological process, cell communication, the cell cycle, cell proliferation, and the defense response (Figure 10.).

6. Expression Validation of Selected Genes

Expression of the selected genes was examined by RT-PCR in the gastric cancer cell lines and tumor tissues. First, we selected 11 genes according to their signal intensity differences between the resistant and sensitive groups in the microarray and according to their functions related to tumors, pharmacology, and paclitaxel (Figure 11.). Then, sqRT-PCR was performed with these 11 genes

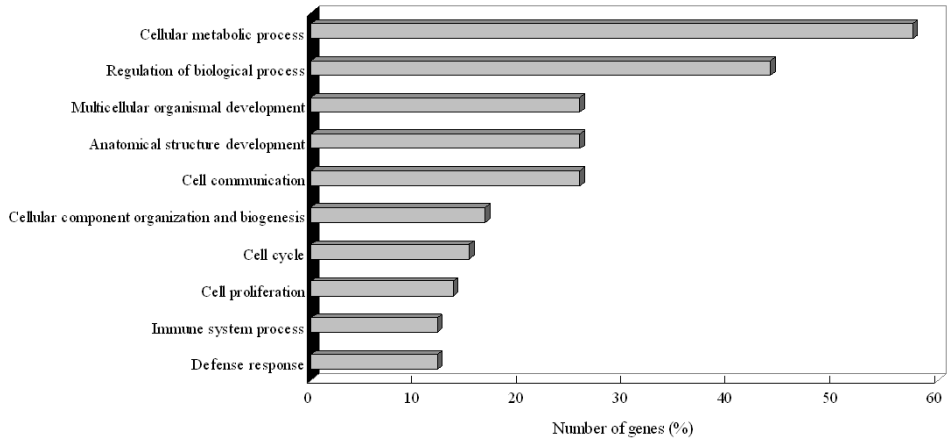

Figure 10. Gene annotation of 115 paclitaxel sensitivity-related genes. Gene annotation was performed using the criteria of over 10% enrichment with statistical significance ($p<0.05$).

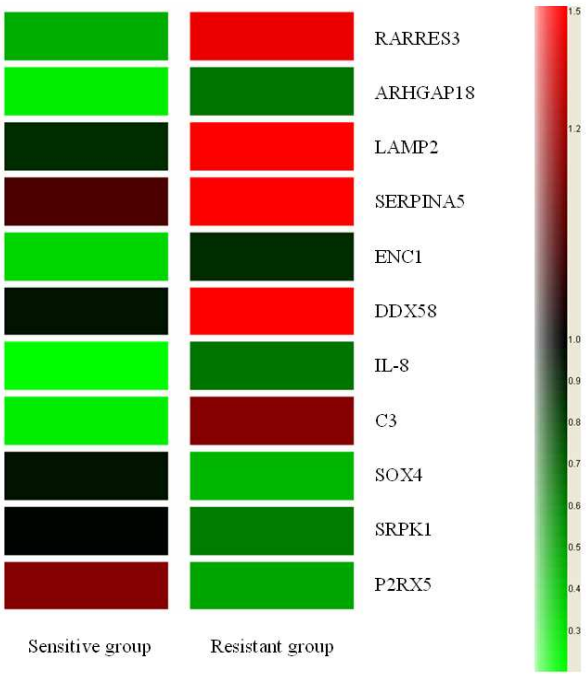

Figure 11. Eleven genes selected for expression validation. These genes were selected according to their different signal intensities in the microarray in resistant and sensitive groups, and for their functions related to tumors, pharmacology, or paclitaxel. Each box represents the mean expression of each gene in the sensitive and resistant groups. The scale bar shows expression level; red denotes high expression and green denotes low expression.

in two resistant gastric cancer cell lines (NCI-N87 and YCC-7), two sensitive gastric cancer cell lines (MKN-45 and SNU-16), resistant gastric tumor tissue (NCI-N87 derived), and sensitive gastric tumor tissue (MKN-45 derived). The results showed that the differential expressions between the two groups were consistent with differences in the microarray (Figure 12.). For further verification, qRT-PCR was conducted with four genes, LAMP2, RARRES3, ARHGAP18, and SOX4, in two gastric cancer cell lines (NCI-N87 and MNK-45) and two gastric tumor tissues derived from NCI-N87 and MKN-45. We calculated a Pearson correlation coefficient between the $\log_2$R/G ratio of the microarray and the $\log_{10}$ relative expression value of qRT-PCR. All genes possessed a high correlation coefficient ($r>0.9$) and the results indicated good correlation between the microarray and qRT-PCR (Figure 13.). The differential expression patterns of the four genes between the two groups were also confirmed by qRT-PCR (Figure 14.).

7. Knockdown of Gene Expression by siRNA

The paclitaxel-resistant gastric cancer cell line, NCI-N87, was subjected to RNA interference specific to two genes, LAMP2 and RARRES3, using siRNA for gene knockdown and functional analysis. We attempted gene knockdown with three siRNAs, each targeting a different site within the gene (Figure 15.). For all three concentrations of siRNA, 2, 10, and 50 nM, the mRNA expression was repressed (Figure 16.). Thus, we decided to use siRNA from site 1, the

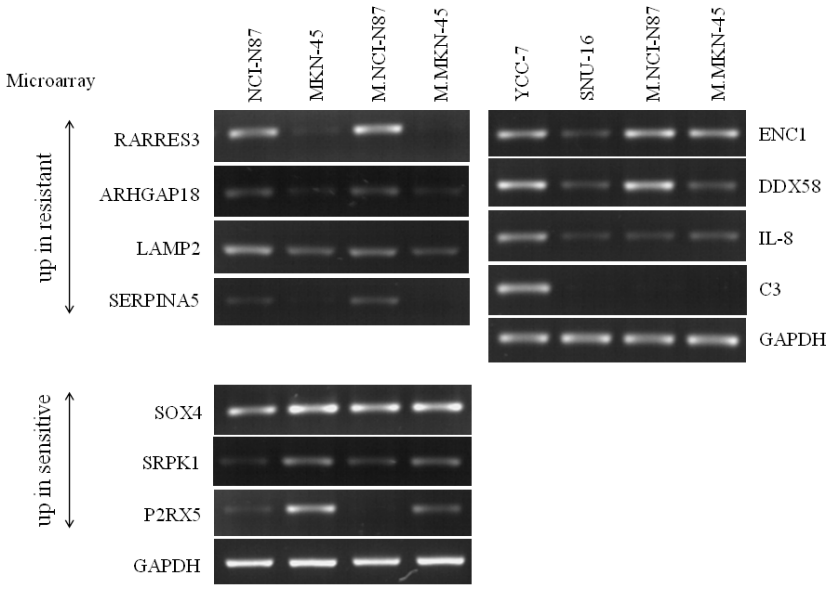

Figure 12. Semi-quantitative RT-PCR. sqRT-PCR was performed with 11 genes in two resistant gastric cancer cell lines (NCI-N87 and YCC-7), two sensitive (MKN-45 and SNU-16) gastric cancer cell lines, and in resistant (M.NCI-N87), and sensitive (M.MKN-45) gastric tumor tissues. These results are consistent with the microarray expression differences.

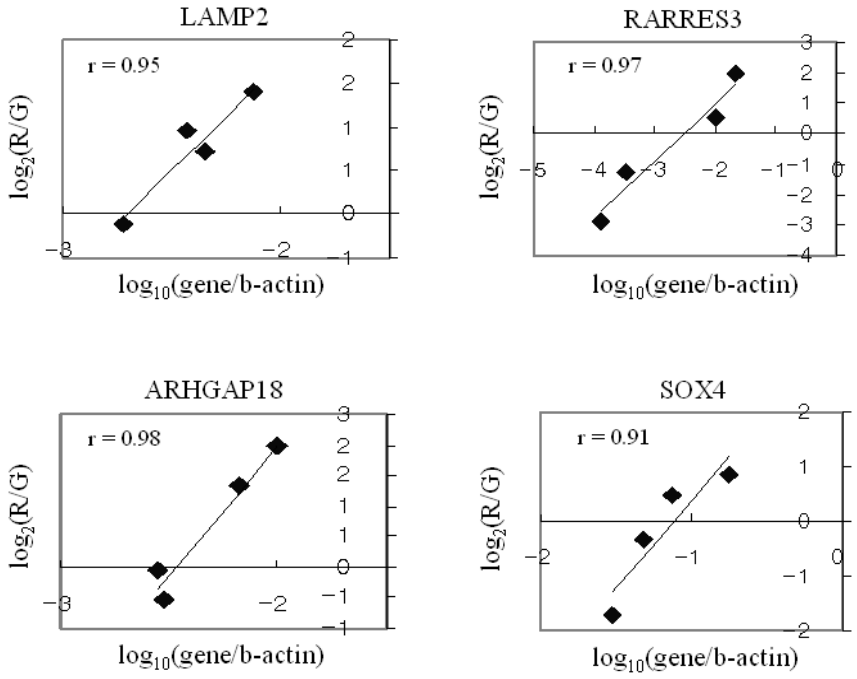

Figure 13. Correlation of microarray and quantitative RT-PCR. qRT-PCR was conducted for four genes: LAMP2, RARRES3, ARHGAP18, and SOX4, in two gastric cancer cell lines (NCI-N87 and MNK-45) and tissues from two gastric tumors derived from NCI-N87 and MKN-45 xenografts. Pearson correlation coefficients (r) between the microarray ($\log_2$(R/G)) and qRT-PCR ($\log_{10}$(gene/b-actin)) were higher than 0.9 in all cases.

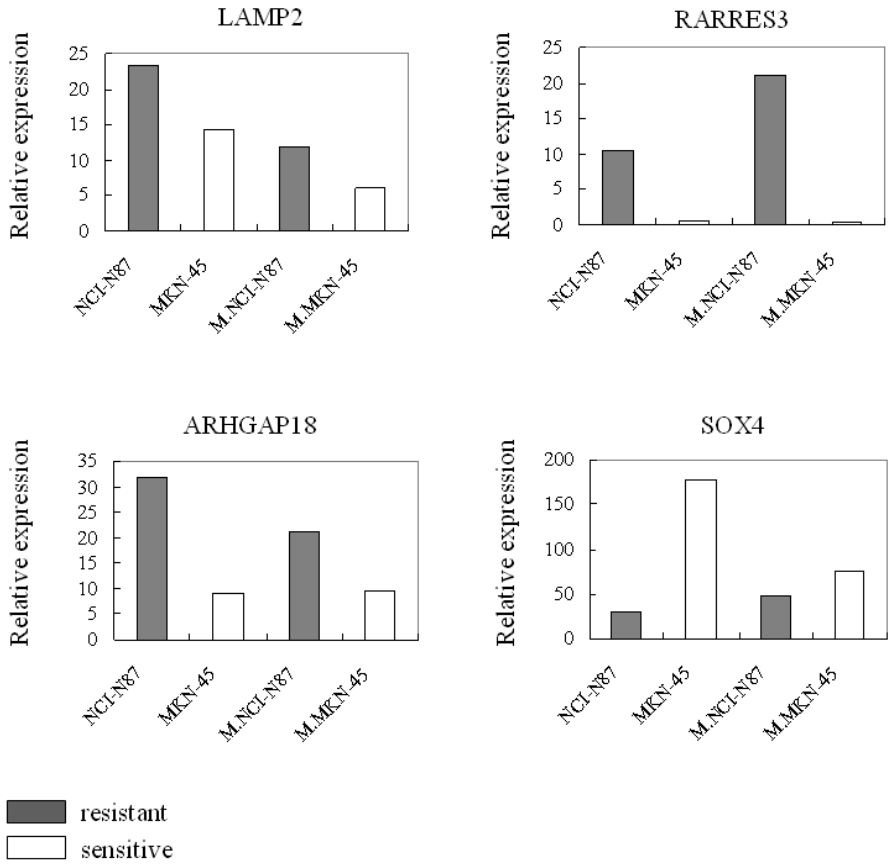

Figure 14. Relative expression by quantitative RT-PCR. The results of qRT-PCR were the same as those for sqRT-PCR in Figure 13 in that differential expression of the four genes between the two groups was seen. M.NCI-N87 and M.MKN-45 represent tumor tissues derived from NCI-N87 and MKN-45 xenografts, respectively.

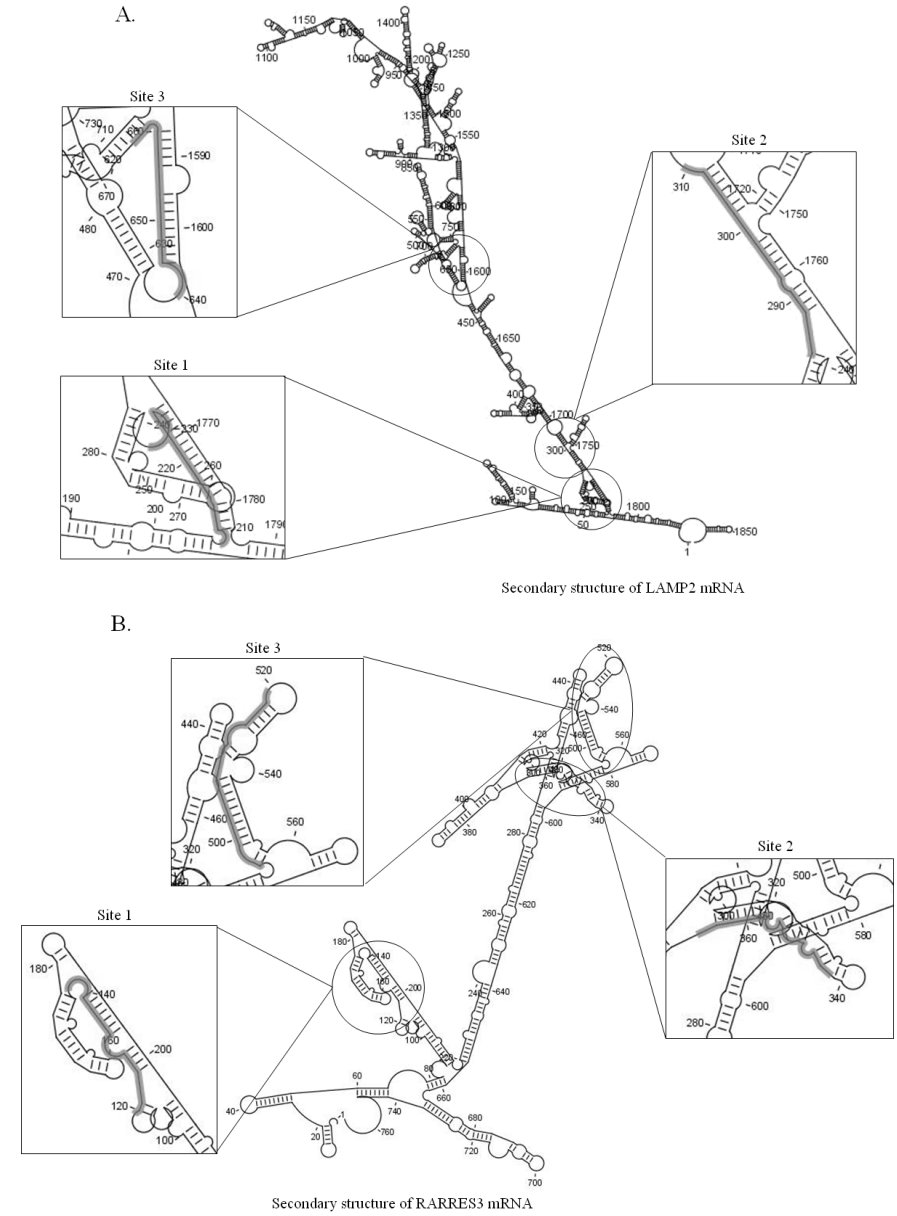

Figure 15. siRNAs targeting mRNA. Three siRNAs targeting different sites within the LAMP2 (A) and RARRES3 (B) mRNA transcripts. Grey lines represent each siRNA.

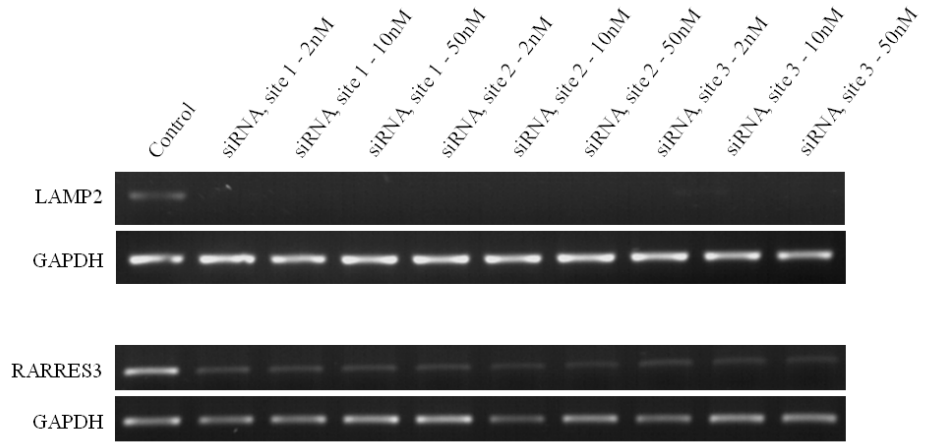

Figure 16. Repressed mRNA expression by siRNA. Three different siRNA concentrations (2, 10, 50 nM) were transfected into NCI-N87 cells for 48 hours and sqRT-PCR was performed. mRNA expressions were repressed in all cases compared with the control (lipofectamine only). The two experiments, LAMP2 and RARRES3, were performed independently.

closest site from the starting region, at a concentration of 2 nM, the lowest concentration, to avoid off-target effects by siRNA[51]. To verify protein repression by siRNA, protein was extracted after 48, 72, 96, and 120 hours of incubation with the siRNA. Compared with control (lipofectamine) and NT siRNA, protein expression was repressed in siRNA treated cells (Figure 17.). The repression of protein expression was evident 48 hours after siRNA transfection After 120 hours, the protein was slightly re-expressed. Thus, subsequent gene knockdown experiments were performed 120 hours after siRNA transfection.

8. Restoration of Paclitaxel Sensitivity-I. Cell Viability Assay

To examine whether a knockdown of selected genes could restore paclitaxel sensitivity, we repressed the expression of LAMP2 and RARRES3, which are over-expressed in resistant cell lines and tissues (Figure 14.), and examined cell viability using MTT assays. In the case of LAMP2, cell viability significantly decreased in LAMP2 knockdown cells after paclitaxel treatment compared with control and NT siRNA-treated cells (Figure 18.). For all concentrations of paclitaxel, this effect was observed with significant differences relative to controls according to the Kolmogorov-Smirnov test ($p<0.001$); this difference was maximized at concentrations of 6 nM or less. This result has very important clinical implications when considering clinically relevant concentrations of paclitaxel in plasma, which are between 5 and 200 nM[52]. A slight inhibition of

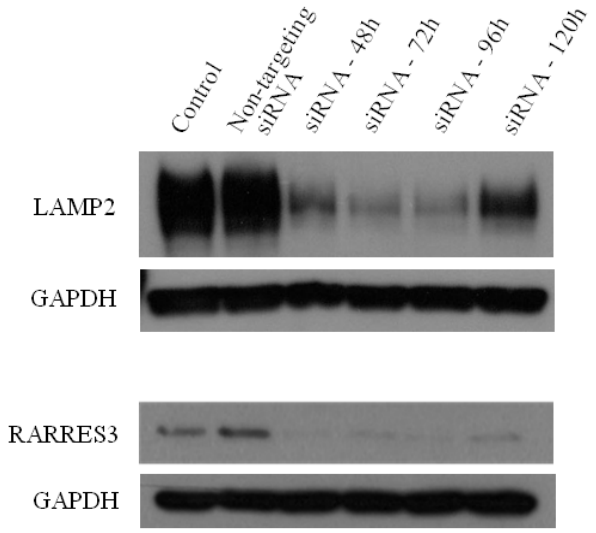

Figure 17. Repressed protein expression by siRNA. Two nanomolar siRNAs were transfected into NCI-N87 cells for 48 to 120 hours and immunoblotting was performed. Protein expression was repressed in all cases compared with control (lipofectamine only) and the NT siRNA-treated groups. Protein expression recovered slightly after 120 hours. The two experiments, LAMP2 and RARRES3, were performed independently.

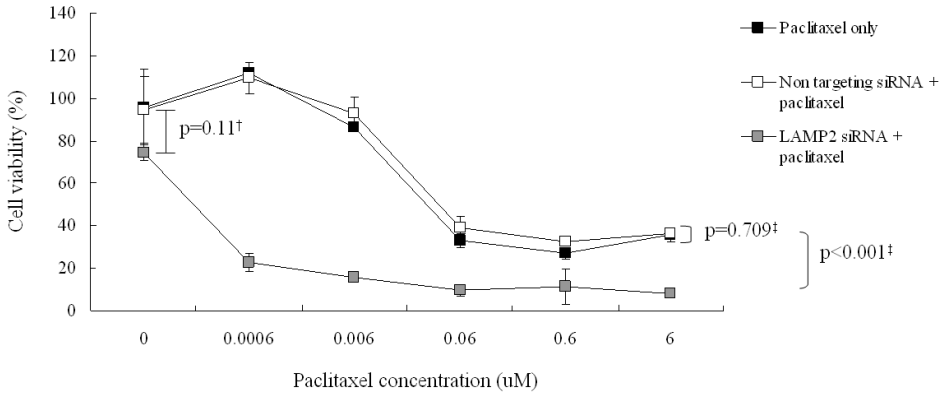

Figure 18. Restoration of paclitaxel sensitivity-I. Cell viability assay. Cell viability was significantly decreased in LAMP2 knockdown NCI-N87 cells after paclitaxel treatment compared with control (paclitaxel only) and NT siRNA-treated NCI-N87 cells. Significantly decreased cell viability was observed at all concentrations of paclitaxel ($p<0.001$), and the difference was maximized at concentrations of 6 nM paclitaxel or less. A slight inhibition of cell viability was observed in the paclitaxel naive LAMP2 knockdown cells, although there was no significant difference compared to control or NT siRNA treated cells ($p=0.11$). † Student's t-test; ‡ Kolmogorov-Smirnov test.

cell viability was observed in the paclitaxel naive LAMP2 knockdown cells; however there was no significant difference compared to control or NT siRNA treated cells ($p=0.11$). RARRES3 analysis showed similar results to LAMP2 (Figure 19.). Although there was no significant difference between RARRES3 knockdown cells and control or NT siRNA cells ($p=0.201$), cell viability was significantly decreased in RARRES3 knockdown cells treated with 6 nM or less paclitaxel ($p<0.05$). This result indicates that knockdown of selected genes restored paclitaxel sensitivity and this effect was evident even when low concentrations of paclitaxel were used.

9. Restoration of Paclitaxel Sensitivity-II. Apoptosis Assay

For apoptosis assays, the paclitaxel concentration was fixed at 6 nM because we wanted to examine the changed apoptotic state in LAMP2 or RARRES3 knockdown cells compared with control and NT siRNA-treated cells in which the apoptotic changes should only be slightly detectable, if not undetectable. After 6 nM paclitaxel treatment for 48 hours, control cells and NT siRNA treated cells did not demonstrate any changes in cell population (Figure 20A, B.). LAMP2 knockdown cells, however, showed significantly increased necrosis and late apoptosis portions in the presence of paclitaxel (Figure 20C.). A bar graph showed that necrosis and late apoptosis were significantly increased by about 4-fold in LAMP2 knockdown cells after treatment ($p<0.005$ and $p<0.0005$, respectively); however control and NT siRNA-treated cells were not

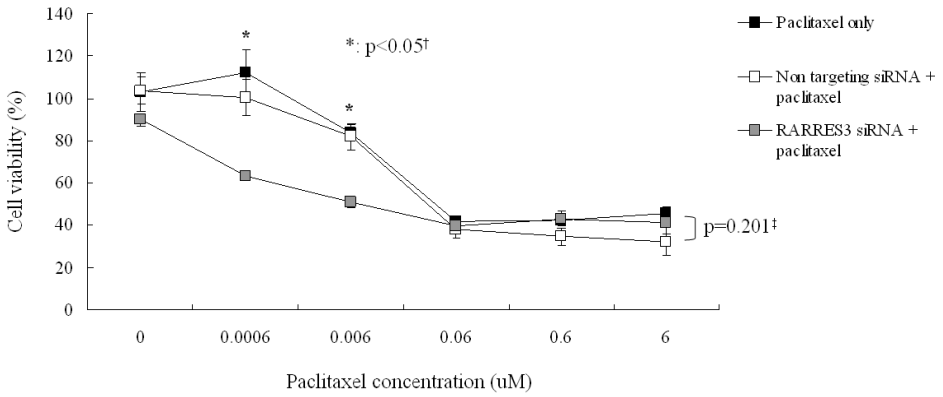

Figure 19. Restoration of paclitaxel sensitivity-I. Cell viability assay. Cell viability was significantly decreased in RARRES3 knockdown NCI-N87 cells after paclitaxel treatment compared with control (paclitaxel only) and NT siRNA-treated NCI-N87 cells. There was no significant difference between RARRES3 knockdown cells and control or NT siRNA cells in the overall concentration of paclitaxel (p=0.201), but cell viability was significantly decreased in RARRES3 knockdown cells treated with 6 nM paclitaxel or less ($p<0.05$). † Student's t-test; ‡ Kolmogorov-Smirnov test.

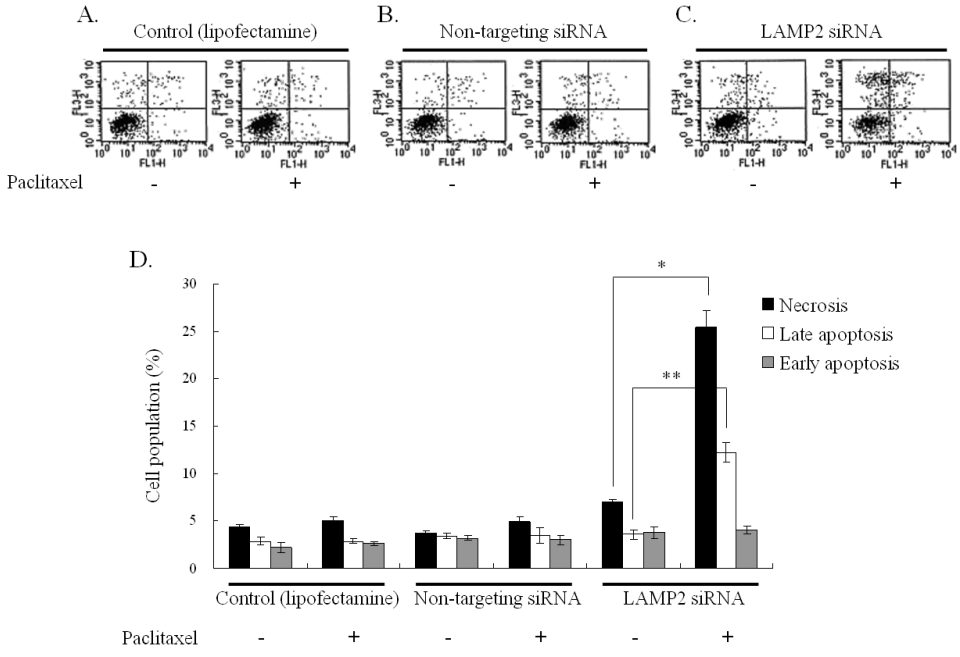

Figure 20. Restoration of paclitaxel sensitivity-II. Apoptosis assay. After 6 nM paclitaxel treatment for 48 hours, NCI-N87 cells were labeled with annexin V-FITC and 7-AAD. A. Control cells treated with lipofectamine only. B. NT siRNA-treated cells. C. LAMP2 knockdown cells. Control cells and NT siRNA-treated cells did not show any changes in cell populations. LAMP2 knockdown cells, however, showed significantly increased necrosis and late apoptosis in the presence of paclitaxel. D. Bar graph showing that necrosis and late apoptosis both significantly increased by approximately 4-fold in LAMP2 knockdown cells whereas there were no significant changes in the NT siRNA-treated cells or control cells. * $p<0.005$; ** $p<0.0005$, Student's t-test.

changed (Figure 20D.). Increased necrosis and apoptosis were also observed in RARRES3 knockdown cells only after paclitaxel treatment ($p<0.005$ and $p<0.0005$, respectively) (Figure 21.). In contrast to LAMP2 knockdown cells, increased early apoptotic cells were detected in RARRES3 knockdown cells with or without paclitaxel treatment; however, the change between paclitaxel naive and paclitaxel-treated RARRES3 knockdown cells was not significantly different ($p>0.05$); this effect was attributed to RARRES3 knockdown. These results revealed that knockdown of selected genes restored paclitaxel sensitivity by increasing necrosis and late apoptosis.

10. G2/M Phase Arrest Effect of Gene Knockdown

We identified the restoration of paclitaxel sensitivity based on cell viability and apoptosis assays. Next, we wanted to examine the G2/M phase cell population that was specifically and directly involved in paclitaxel mechanism in intact cells. After treatment with 6 nM paclitaxel for 24 hours, control and NT siRNA-treated cells showed a slight decrease in the G1 phase and a counter increase in the sub-G1 phase, but no changes in the G2/M phase (Figure 22A, B.). However, in LAMP2 knockdown cells, the G2/M phase population increased by over 2-fold; this was accompanied by a decrease in the G1 phase population (Figure 22C.). In this case, the ratio of populations in G1/G2/M phases was reversed compared with control and NT siRNA. Thus, we reached the conclusion that knockdown of LAMP2 restored paclitaxel sensitivity not only by increasing

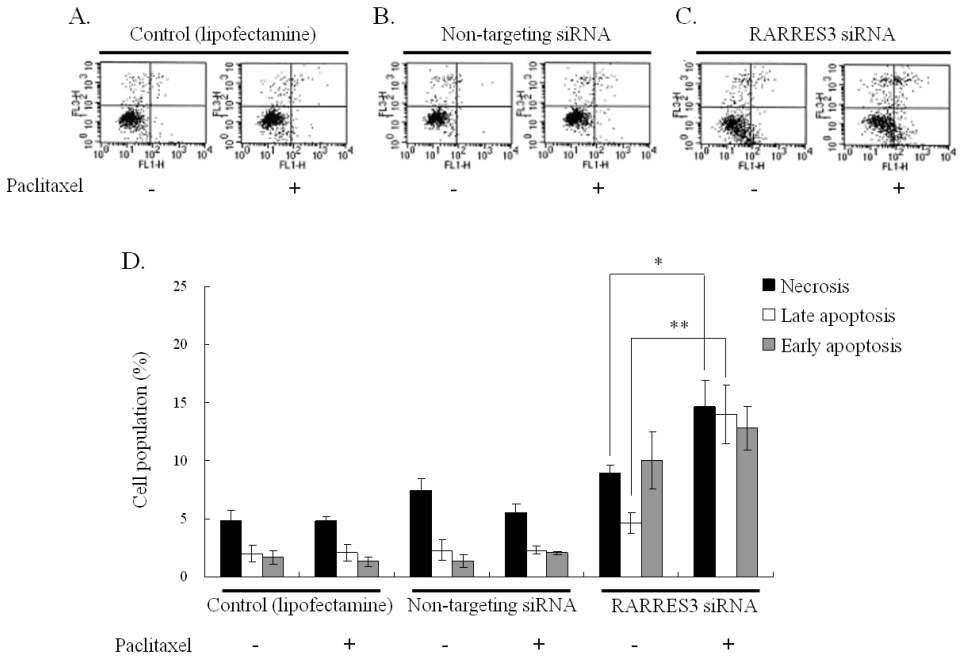

Figure 21. Restoration of paclitaxel sensitivity-II. Apoptosis assay. After 6 nM paclitaxel treatment for 48 hours, NCI-N87 cells were labeled with annexin V-FITC and 7-AAD. A. Control cells treated with lipofectamine only. B. NT siRNA treated cells. C. RARRES3 knockdown cells. Control cells and NT siRNA treated cells did not show any changes in cell populations. RARRES3 knockdown cells, however, showed significantly increased necrosis and late apoptosis in the presence of paclitaxel. D. Bar graph showing that necrosis and late apoptosis both significantly increased in RARRES3 knockdown cells whereas there were no significant changes in the NT siRNA-treated cells or control cells. * $p<0.005$; ** $p<0.0005$, Student's t-test.

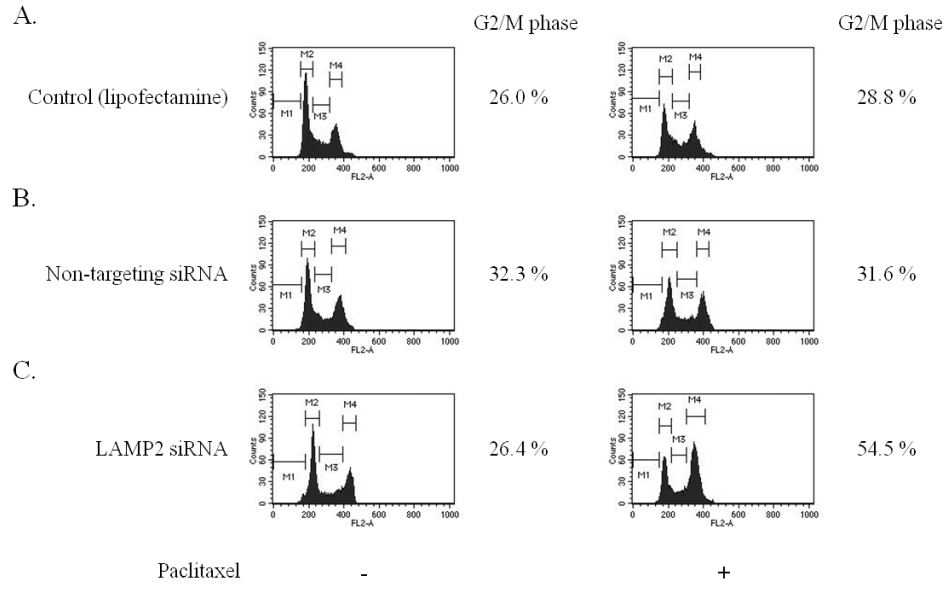

Figure 22. Cell cycle arrest analysis. After treatment with 6 nM paclitaxel for 24 hours, NCI-N87 cells were labeled with PI. A. Control cells treated with lipofectamine only. B. NT siRNA-treated cells. C. LAMP2 knockdown cells. Control and NT siRNA-treated cells showed a slight decrease of the G1 phase and a counter increase of the sub-G1 phase but no changes in the G2/M phase. In LAMP2 knockdown cells, the G2/M phase population increased by over 2-fold with a concomitant decrease in the G1 phase population.

necrosis and late apoptosis, but also by arresting the cell cycle in the G2/M phase. RARRES3 knockdown, on the other hand, did not affect G2/M arrest; based on this we hypothesized that different genes influence paclitaxel sensitivity in different ways.

IV. DISCUSSION

We selected paclitaxel resistant and sensitive cell lines from 78 various human cancer cell lines according to their chemosensitivity values (IC_{50}s). Staunton et al. first applied this algorithm with 60 human cell lines (the NCI-60)[5]. In their study, they used the following criteria for cut-off values: 0.8 standard deviations, which divided the 60 cell lines into resistant, sensitive, and intermediate groups, the latter which was excluded from gene selection. We investigated a larger number of cell lines, 78 human cancer cell lines, and applied one standard deviation of the IC_{50} as a cut-off. This criterion was more strict with respect to excluding intermediates. After successful internal validation, with a prediction accuracy of 93% for the resistant and sensitive cell lines, we performed in vivo prediction as well as expression and functional validation.

Using 115 selected genes, we performed gene annotation to narrow down the genes for validation. Among the overexpressed genes in the resistant cell lines, IL-8, C3, SERPINA5, IFN-α, RARRES3, ENC1, LAMP2, ARHGAP18, and DDX58 were selected first. It bas been reported that IL-8 is over-expressed in paclitaxel resistant cell lines[53] and that increasing IL-8 concentration correlates with a poor initial response to paclitaxel chemotherapy[54]. C3 and SERPINA5 are members of the ‘complement and coagulation cascades’ pathway stimulated by the chemotactic effect (IL-8). RARRES3 and its stimulator, IFN-α, were co-up-regulated in the resistant group and these genes are thought to be regulators of cytokine expression and tumor growth[55]. ENC1 was also selected because its

over-expression has been examined during carcinogenesis[56]. ARHGAP18 and DDX58 were also selected because of their highly differential expressions and not because of their functions. Among these genes, we were particularly interested in LAMP2, a major component of the lysosome, because LAMP2 has been implicated in the elimination of foreign materials, including drugs, by the lysosome. Several researchers have previously reported that LAMP2 and the lysosome are associated with drug resistance and cell death in cancer cells. Lysosomal permeabilization induces tumor cell death by break-down of the lysosomal membrane and the release of cathepsin, lysosomal hydrolase[57,58]. Recent research has shown that lysosomes perform important functions in some forms of apoptotic cell death[58-60]. Several studies have suggested that lysosome-associated apoptosis is induced by a number of conventional anticancer drugs, including etoposide, cisplatin, and 5-fluorouracil[61]. For these reasons, the lysosomal death pathway is attracting considerable interest as a drug target and as a possible source of combination anticancer drug therapy.

In sensitive cell lines, we selected SRPK1, SOX4, SEPT6, and P2RX5 as up-regulated genes. SRPK1 is known for a cisplatin sensitivity gene, the inactivation of which can lead to cisplatin resistance in a human ovarian cancer cell line[62]. Research has shown that the inhibition of SRPK1 causes 4-fold resistance to cisplatin. Resistance to platinum-containing chemotherapy in testicular germ cell tumors was also reported to be associated with the down-regulation of the protein kinase SRPK1[63]. Ahn et al. published results indicating that SOX-4 is a positive regulator of apoptosis induced by prostaglandin,

followed by caspase-1[64]. In relation to microtubules, which are a direct target of paclitaxel, SEPT6 has been reported that suppression of septin expression causes increased microtubule stability via MAP4 (microtubule binding protein) fragment binding to microtubules[65]. Based on its differential expression pattern in sensitive cell lines compared to resistant cell lines, P2RX5 was also selected. When we performed expression validation with quantitative RT-PCR for these selected genes, however, IFN-α and SEPT6 did not show differential expression patterns in the NCI-N87 and MKN-45 cell lines.

In 2006, Potti et al. reported several gene sets that could predict responses to anticancer drugs, including paclitaxel[66]. There were 36 paclitaxel predictors; however, none of these were found in the 115 genes used in this study; this discrepancy may be due to the use of different cell lines for gene selection. Potti et al. used the NCI-60 panel, which does not contain any gastric cancer cell lines, and there was no prediction analysis with gastric cancer specimens. In our research, among the 78 major cell lines, 23 were gastric cancer cell lines. Therefore, it is likely that the 115 genes identified in this study could be more useful for predicting paclitaxel sensitivity in gastric cancers. Indeed, we were able to accurately predict the sensitivity of gastric cancer *in vivo*.

By performing functional studies with two representative genes (LAMP2 and RARRES3), we confirmed that knockdown of selected genes that are over-expressed in resistant cell lines restored paclitaxel sensitivity *in vitro*. For both LAMP2 and RARRES3 knockdowns, we observed slightly reduced cell viability in siRNA transfected cells, although there were no significant

differences in viability when compared with control cells (Figure 18, 19.). This result is consistent with a previous report in which knockdown of LAMP2 induced a low level (10%) of apoptosis[67]. RARRES3 may function to regulate tumor growth, although this study did not directly evaluate this possibility. The possible mechanism by which LAMP2 knockdown restores paclitaxel sensitivity is thought to be a loss of lysosomal function or a break in the lysosomal membrane. Loss of lysosomal function by LAMP2 knockdown could disturb the elimination of intracellular paclitaxel by hydrolysis or exportation[68]. A break in the lysosomal membrane by LAMP2 knockdown could cause permeability of the lysosomal membrane, leading to cathepsin release from the lysosome [57,58], which could induce cell death. These events may have been the mechanism underlying restored paclitaxel sensitivity in resistant gastric cancer cell lines. Restoration of paclitaxel sensitivity was also observed in RARRES3 knockdown cell lines. Increased RARRES3 expression is known to decrease cell growth through the MAPK signal pathway[69-71]. Conversely, decreased RARRES3 could increase cell growth through rapid cell cycle progression, giving cells more of a chance to come into contact with paclitaxel when compared with RARRES3-expressing cells. Therefore, the restoration of paclitaxel sensitivity could also occur in RARRES3 knockdown and resistant gastric cancer cell lines.

In this study, we selected 115 genes related to paclitaxel sensitivity using a large number of human cancer cell lines and the predictive power of the 115 genes was confirmed by *in vivo* gastric cancer xenografts. Using this gene set,

further studies including clinical validation studies should be performed to assess the ability of this set of genes to predict the response to paclitaxel in chemo-naive gastric cancer patients. In this study, we also revealed that knockdown of a single gene could restore paclitaxel sensitivity in a paclitaxel-resistant cell line. Following the process we outlined in this functional study, more candidate genes that may also act as chemo-sensitizers of paclitaxel are expected to be found.

V. CONCLUSION

To identify genes related to paclitaxel chemosensitivity in gastric cancer, we first selected 115 paclitaxel-related genes *in vitro* using 78 human cancer cell lines of various tissue origins. Second, *in vivo* prediction of paclitaxel sensitivity was performed with human gastric cancer xenografts derived from the resistant and sensitive gastric cancer cell lines, NCI-N87 and MKN-45, respectively. The gene set comprising the 115 selected genes was found to correctly predict *in vivo* chemosensitivity. Thus we concluded that these 115 genes might be predictive markers for paclitaxel chemotherapy in gastric cancer. Third, to certify whether these genes could change paclitaxel sensitivity, the expression of LAMP2 and RARRES3 was repressed by siRNA, and subsequently, cell viability assays, apoptosis assays, and cell cycle arrest analyses were performed. Knockdown of the genes restored paclitaxel sensitivity by increasing necrosis, late apoptosis, and cell cycle arrest at the G2/M phase. In conclusion, these selected genes may act as not only predictive markers, but also as chemo-sensitizers of paclitaxel. Clinical validation will be required to confirm whether these genes are applicable to chemo-naive gastric cancer patients.

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Abstract (in Korean)

위암에서의 paclitaxel 감수성과 관련된 유전자군

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정 재 준

암세포의 유사분열기 동안 미세소관의 해중합화 과정을 억제함으로써 세포사멸을 일으키는 항암제인 paclitaxel은 다양한 암종에서 사용되고 있다. Paclitaxel의 저항성은 넓은 범위에서 연구되어 왔지만, 그 메커니즘은 MDR1과 tumulin에 국한되어 왔다. 따라서 본 연구에서는 high-throughput 방법을 이용하여 새로운 paclitaxel 감수성 관련 유전자를 찾았고, 이 유전자들의 기능을 paclitaxel 감수성과 관련된 측면에서 증명하였다. 먼저 MTT-assay와 oligonucleotide microarray를 이용하여 78개 인간 암세포주의 항암제감수성과 유전자 발현 정도를 각각 구축하였다. 이 두 정보를 통합함으로써 paclitaxel 저항성, 민감성을 나타내는 집단 사이에서 발현의 차이를 보이는 paclitaxel 감수성 관련 유전자 군을 선별하였다. Leave-one-out cross-validation (LOOCV) 방법으로 내부 검증을 수행하였고, 위암세포주를 이용하여 만든 암조직의 항암제감수성을

support vector machine (SVM)으로 예측하였다. 선별된 유전자들의 위암세포주에서의 기능적 평가를 위해서 siRNA를 이용하여 유전자들의 발현을 억제시킨 뒤 세포 생존능력 분석, apoptosis 분석, 세포주기 분석을 수행하였다. 본 연구에서는 paclitaxel 감수성과 관련된 115개 유전자를 선별하였고, LOOCV는 93%의 높은 예측율을 보였다. SVM 결과에서도 선별된 유전자들은 100% 예측율을 나타냈고, 이 결과를 토대로 선별된 유전자들은 paclitaxel 항암요법에 있어서 예측인자가 될 수 있음을 보였다. Paclitaxel 저항성 위암세포주 (NCI-N87)와 민감성 위암세포주 (MKN-45), 그리고 이 세포주들로 만든 암조직에서 선별된 유전자들의 발현정도를 quantitative, semi-quantitative RT-PCR 기법을 이용하여 증명하였다. 선별된 유전자 중 LAMP2와 RARRES3에 대해서는 NCI-N87 세포주에서 기능적 연구를 수행하였다. 먼저 siRNA를 이용하여 유전자의 발현을 억제시킨 결과 두 유전자의 발현이 억제됨을 확인하였다. 이어서 수행한 세포 생존능력 분석, apoptosis 분석, 세포주기 분석 결과, 두 유전자의 발현 억제가 necrosis, 늦은 apoptosis, 세포주기의 G2/M기 억류 등을 통해서 paclitaxel의 감수성을 회복시킴을 보였다. 결론적으로 선별된 유전자들은 항암치료를 받지 않은 위암 환자에 있어서, paclitaxel 항암요법에 대한 예측인자가 될 수 있을 뿐만 아니라 paclitaxel의 항암제 민감인자가 될 수 있을 것으로 생각된다.

핵심되는 말: paclitaxel 감수성, 예측인자, 약물유전체학, 위암