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Establishment of New Intraperitoneal Paclitaxel-Resistant Gastric Cancer Cell Lines and Comprehensive Gene Expression Analysis

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Abstract. *Background: Intraperitoneal (i.p.) chemotherapy with paclitaxel is a potential therapeutic modality for patients with peritoneal metastasis of gastric cancer. To overcome paclitaxel resistance, which is a major clinical problem with this modality, prediction of i.p. paclitaxel resistance is critically important. Materials and Methods: We developed three new i.p. paclitaxel-resistant cell lines from parental gastric cancer cell lines by in vivo selection method using i.p. paclitaxel chemotherapy. With these cell lines, we performed gene expression profiling analysis to select up-regulated genes in i.p. paclitaxel-resistant cells and validated the genes with clinical samples. Results: We successfully isolated nine up-regulated genes in i.p. paclitaxel-resistant cell lines compared with parental cells by microarray analysis, followed by confirmation with quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Among these, we identified four genes, namely kinesin family member 23 (KIF23), ERBB2 interacting protein (ERBB2IP), ATPase family, AAA domain containing 2 (ATAD2) and PHD finger protein (PHF19) as candidate genes for paclitaxel resistance after validation with clinical samples derived from responders and non-responders to paclitaxel treatment. Conclusion: These i.p. paclitaxel-resistant cell lines are ideal models for understanding the mechanism of resistance to i.p. paclitaxel and development of a new therapeutic modality. Four up-regulated genes may be potential new predictive markers for resistance to i.p. paclitaxel in patients with peritoneal metastasis of gastric cancer.*

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Although the survival of patients with gastric cancer has improved due to the development of new diagnostic tools and therapeutic approaches, such as molecular targeting therapy, it remains one of the leading causes of cancer death in Japan, as well as in East Asian and some Western countries. Peritoneal metastasis accounts for 40-60% of recurrence after curative surgery in patients with gastric cancer and is therefore the most important prognostic factor (1, 2). It causes not only cancer death, but also intestinal obstruction and malignant ascites formation, which remarkably restrict the quality of life (QOL) of the affected patients. Despite advances in therapeutic modalities for peritoneal metastases, such as combination chemotherapy (3), a standard treatment has not yet been established because advanced peritoneal deposits are refractory to various chemotherapeutic agents (4). Molecular targeting therapy using small molecular inhibitors and therapeutic monoclonal antibody is one potential alternative for conventional chemotherapy. To date, however, only a few preclinical studies and clinical trials for peritoneal metastasis of gastric cancer have been reported (5).

Paclitaxel is a mitotic inhibitor that bindsβtubulin and thereby stabilizes microtubules, interferes with breakdown of microtubules and consequently inhibits the progression of the G_2/M stage during cell division. Paclitaxel is one of the most active anticancer agents, effective against a broad range of epithelial cancer types, including breast, ovarian and gastric cancer. Because of its peculiar pharmacological characteristics, paclitaxel is maintained at substantially high intraperitoneal concentration for a prolonged period when delivered intraperitoneally, which results in a remarkable anti-metastatic effect on peritoneal metastasis of gastric cancer in preclinical models (6). Intraperitoneal

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administration of paclitaxel has been shown to be effective and safe for peritoneal dissemination of ovarian cancer in clinical settings (7). Kodera *et al*. previously demonstrated the safety and effectiveness of *i.p*. paclitaxel monotherapy against gastric cancer in a phase I clinical trial (8). Furthermore, Ishigami *et al*. conducted a one-arm single-institutional phase I and phase II clinical trial and showed the efficacy of a regimen combining *i.p*. paclitaxel with established systemic chemotherapy of S-1 plus intravenous paclitaxel therapy (9, 10). These clinical data suggest that paclitaxel therapy is a promising therapeutic modality for patients with peritoneal metastasis of gastric cancer.

A clinically important issue is the fact that these patients with peritoneal metastasis not infrequently show resistance to paclitaxel therapy. Recently, several investigators have reported the molecular mechanisms or markers for tumor cell resistance to paclitaxel, such as overexpression of adenosine triphosphate(ATP)-binding cassette transporters (11), β-tubulin isotypes (12) and miRNA such as miR-34 (13). Paclitaxel-resistant cell lines used in these studies were mostly derived from ovarian cancer and were established *in vitro* through stepwise selection in increasing drug concentrations (14). Recently, Okugawa *et al*. reported that paclitaxel-resistant ovarian cancer cell line established *in vitro* selection method exhibited high paclitaxel resistance in *in vitro* culture condition, but lost this resistance or tumorigenicity in a transplanted tumor model in mice (15), indicating some difficulty in establishing a clinically suitable paclitaxel-resistant model by the *in vitro* selection method. Furthermore, in gastric cancer, there is substantially no paclitaxel-resistant cell lines established by either *in vitro* or *in vivo* selection.

In the present study, we newly established three paclitaxel-resistant gastric cancer cell lines by the *in vivo* selection method. Using these parental and resistant cell lines, we conducted expression profiling analysis to explore new predictive markers for paclitaxel resistance in gastric cancer.

Materials and Methods

Reagents, cell lines and animals. Paclitaxel was purchased from Bristol-Myers Squibb Japan (Tokyo, Japan). GCIY and MKN28 cell lines were obtained from the RIKEN Cell Bank (Tsukuba, Japan). GPM-1 cell line was established previously in our laboratory (Aichi Cancer Center Research Institute, Japan) (16). GCIY and GPM-1 cell line are a poorly-differentiated human gastric carcinoma cell lines established from ascites of a gastric cancer patient, while MKN28 is a differentiated type gastric cancer cell line. These cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) with 100 units/ml penicillin and 100 units/ml streptomycin sulfate and cultured in a humidified 5% CO_2. Six-week-old male athymic nude mice of the KSN strain were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and housed under specific pathogen-free condition. All experiments were carried out with the approval of the Institutional Ethical Committee for Animal Experiment of Aichi Cancer Center Research Institute.

Establishment of i.p. paclitaxel-resistant cell lines. Parental tumor cell suspensions (5×10^6 cells) were injected into the peritoneal cavity of mice. Mice bearing peritoneal metastases were treated with weekly *i.p*. paclitaxel (25 mg/kg/week) six times from three days after inoculation of tumor cells. Autopsy was performed after the last paclitaxel administration, and small residual metastatic nodules were removed and cultured with Dispase (Godo Shusei, Tokyo, Japan) as described previously (17). After repeating this procedure two or three times, GCIY-PTXR3, GPM1-PTXR2 and MKN28-PTXR3 cell lines were obtained.

In vitro cell growth and chemo-sensitivity assay. Cells were plated at 1×10^4 cells/ 96-well plastic plate in DMEM supplemented with 10% FBS. The number of viable cells was counted with a hemocytometer in triplicate every 24 hours after seeding for three days. For chemosensitivity tests, cells were plated in 96-well plates at 1.0×10^4 cells per 200 μl of medium containing 10% FBS. After incubation for 24 hours at 37˚C, the medium was replaced by fresh medium with or without different concentrations of paclitaxel. Another 72 hours later, the number of viable cells was counted with a hemocytometer in triplicate.

Microarray analysis. In order to search for candidate genes related to paclitaxel resistance, we performed a comprehensive DNA microarray analysis using Human 25K Oligo DNA chip according to the protocol for the manufacture of the 3D-GeneTM (Toray Industries, Inc., Tokyo, Japan) and selected genes whose expression level in the resistant cell lines was about twofold increased compared with the parental cells. Total RNA extracted from each cell line by RNeasy Mini kit (Qiagen, Hilden, Germany) was amplified using the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Applied Biosystems, CA, USA). aRNA (1 μg) from gastric cancer cell lines was labeled with Cy5 and hybridization of each parental and resistant gastric cancer cell line was performed. The hybridized DNA microarray was scanned for Cy5 fluorescence by DNA microarray scanner (3D-Gene Scanner; Toray Industries, Inc., Tokyo, Japan), and the fluorescent image data were converted into signal intensity using 3D-Gene Extraction software (Toray Industries, Inc.). Each DNA microarray was globally normalized using the median of a reliable spot. After global normalization, the MA plot was made to visualize the normalized microarray data and to identify genes for statistical analyses, where M is the normalized data ratio (=log2Cy5-log2Cy3) and A is the average normalized data for a dot in the plot.

Clustering analysis. A heat-map of gene expression in the parental and paclitaxel-resistant cell lines was constructed by hierarchical cluster analysis using Cluster 2.0 software, and the results were displayed with the TreeView program (http://rana.lbl.gov/eisen/). Red, black, and green indicate a fold-change expression level above, at, and below 1, respectively.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). cDNA was synthesized from total RNA using random hexanucleotide primers and SuperScript II RNase H-reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA).

Table I. *List of candidate genes selected for i.p. paclitaxel resistance in this study*

Symbol	Gene names	Feature
KIF23	kinesin family member 23	A kinesin-like motor protein that localizes to the interzone of mitotic spindles in the nucleus composed by microtubles
ERBB2IP	Erbb2 interacting protein	It binds to the ERBB2 protein and regulates ERBB2 function and membrane localization. It also locates in the nucleus of the mitotic cells with increase at G_2/M phase
ATAD2	ATPase family, AAA domain containing 2	AAA nuclear coregulator cancer-associated protein which possesses an AAA-type ATPase domain that recognize specifically acetylated histones such as H3K14ac
PHF19	PHD finger protein 19	Human Polycomb-like protein which binds trimethylated histone H3 Lys36 as a repressive chromatin modifier

Single-step real-time RT-PCR for each mRNA was performed by Universal Probe Library system (Roche, Rotkreuz, Switzerland) using specific primers and TaqMan probe on the LightCycler instrument (Roche, Mannheim, Germany). *GAPDH* was analyzed as internal control. The sequences (5'-3') of the primers and TaqMan probes (Universal Probe Library probe) for the candidate genes used in this study are follows: *KIF23* sense primer 5'-GCCGAGAGCTACACGTTCA-3', antisense primer 5'-GACCGG TGCATCTTCAAA-3', probe no. 80; *ERBB2IP* sense primer 5'-CTCTGTGGGGACTTCAACG-3', antisense primer 5'-TGGGTG TCAGCTTGGTGTT-3', probe no. 55; *ATAD2* sense primer 5'-GCAGCATTACCTGCTCTACGTT-3', antisense primer 5'-GCTTG ATAAGCTGATGCTGTAATTT-3', probe no. 71; *PHF19* sense primer 5'-AAAGTGTGGCTGCCAAGAAC-3', antisense primer 5'-AGCCTCAGAGAGGTCAGCAA-3', probe no. 16. Amplification with RT-PCR was performed by 40 cycles at 95°C (10 s.) for denaturation, 60°C (30 s.) for annealing and 72°C (1 s.) for extension.

Validation in clinical samples. We examined time to treatment failure (TTF) of 37 patients with gastric cancer treated with paclitaxel in our groups' hospitals. TTF indicates the period from the start until the stop of the therapy because of disease progression. Among these, seven patients with long TTF (range=210-772 days) were selected as responders and five patients with short TTF (range=26-77 days) as non-responders. The average age of responders and non-responders was 56 and 66 years and male/female ratio was 5/2 and 5/0, respectively. Disease stage (III/IV) of the responders and non-responders was 3/4 and 2/3, and histology (diffuse/intestinal type) was 7/0 and 3/2, respectively. To validate the 78 candidate genes selected by microarray analysis, we performed qRT-PCR analysis of surgically resected, fresh primary gastric cancer tissues from affected patients and compared the gene expression between responders and non-responders.

In vivo studies. Each parental and paclitaxel-resistant cell line (5.0×10^6 cells/0.3 ml medium) were injected into mice intraperitonealy. Those mice that were inoculated with each cell line were then divided into a treatment group and a control group. In the treatment group, mice were treated with five weekly *i.p.* administrations from two days after the inoculation of each cell line. In the control group, mice underwent *i.p.* administration of the vehicle five times. Survival time was compared between the treatment group and control group for each type of cell.

Statistical analysis. Survival period was analyzed by the Kaplan-Meier method and compared with the log-rank test. For data on *in vitro* experiments, statistical comparisons among groups were performed by applying the Student's *t*-test.

Results

Establishment of i.p. paclitaaxel-resistant gastric cancer cell lines. We established three *i.p.* paclitaxel-resistant sublines (GCIY-PTXR3, GPM1-PTXR2 and MKN28-PTXR3) from parental cell lines (GCIY, GPM1 and MKN28) by two to three *in vivo* selections. Since MKN28 cells are more sensitive to *i.p.* paclitaxel treatment than GCIY and GPM1 cells, and MKN28 tumor-bearing mice were found to become almost tumor-free after the same *i.p.* paclitaxel treatment, we reduced the number of treatments from six to three (Figure 1A).

Growth rates of GCIY-PTXR3 and GPM1-PTXR2 cells were significantly increased compared with parental cells, and their morphology also changed from flattened to more round shape, with less cohesion. In contrast, differences in the growth and morphology between MKN28-PTXR3 and parental cells were much less than in the other two pairs (Figure 1B and 2A).

Sensitivity to paclitaxel. The sensitivity to paclitaxel was compared between parental cells and resistant cells both *in vitro* and *in vivo*. *In vitro* growth inhibition of GCIY-PTXR3 and GPM1-PTXR2 cells by paclitaxel was significantly lower than that of parental cells. However, the growth inhibition of MKN28-PTXR3 cells by paclitaxel was only observed at high paclitaxel concentration (10 μM) (Figure 2B), indicating strong paclitaxel resistance in GCIY-PTXR3 and GPM1-PTXR2 cells, and weak resistance in MKN28-PTXR3 cells.

As an *in vivo* paclitaxel sensitivity test, we next conducted Kaplan–Meier survival analyses using mice-bearing peritoneal metastases with and without *i.p.* paclitaxel treatment. In parental cells, mice with *i.p.* paclitaxel treatment had a significantly much better prognosis than those of non-treated control mice (Figure 3A). In contrast,

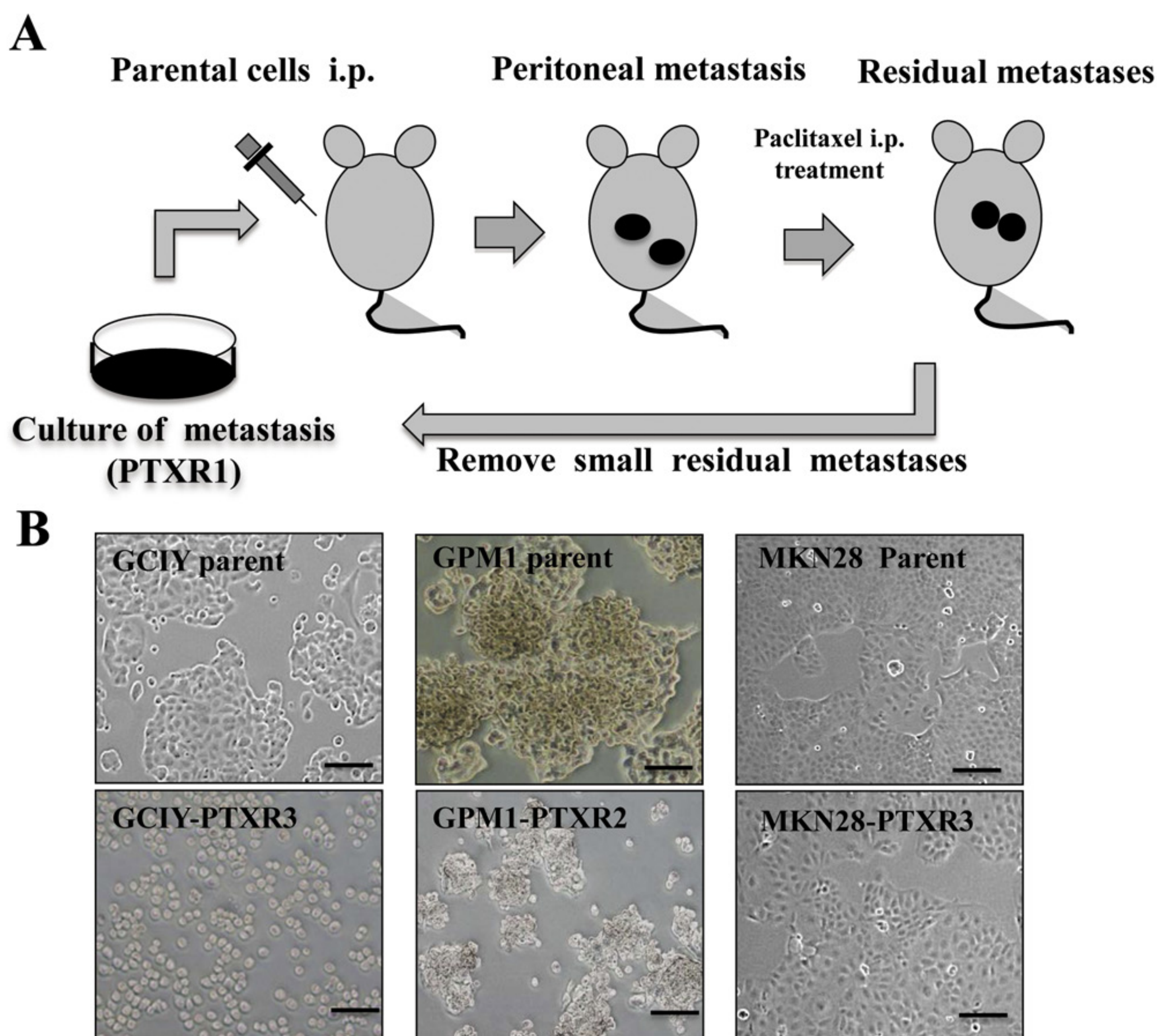

Figure 1. *Isolation and characterization of paclitaxel-resistant cells. Schematic representation of isolation method by in vivo selection. Mice given an injection of 5×10^6 cells were administered paclitaxel at a dose of 25 mg/kg/day or the vehicle starting from day 2 post-injection of tumor cells (A). Phase contrast photomicrographs of parental and paclitaxel-resistant sublines (B). Bar=50 μm.*

survival benefits obtained from *i.p.* paclitaxel treatment in paclitaxel-resistant cells was less than in parental cells, especially for GCIY-PTXR3 cells (Figure 3B), further confirming *i.p.* paclitaxel resistance of paclitaxel-resistant sublines *in vivo*. The relative intensity of *i.p.* paclitaxel resistance was GCIY-PTXR3> GPM1-PTXR2> MKN28-PTXR3.

Differentially expressed genes associated with i.p. paclitaxel resistance. Genes which were approximately twofold up-regulated in resistant cells compared with parental cells were first selected by MA plot analysis (Figure 4A). Using this analytical method, we further selected the genes overexpressed in common to at least two resistant cell lines compared with their parental cell lines. The resultant Venn diagram (A=46 genes, B=19 genes, C=11 genes, and D=2 genes, area) showed an overlap of 78 (664, ratio>1.5) up-regulated genes in three pairs of the parental and paclitaxel-resistant cells (A+B+C+D) (Figure 4B). Among the 78 genes, seven were found to be expressed significantly more

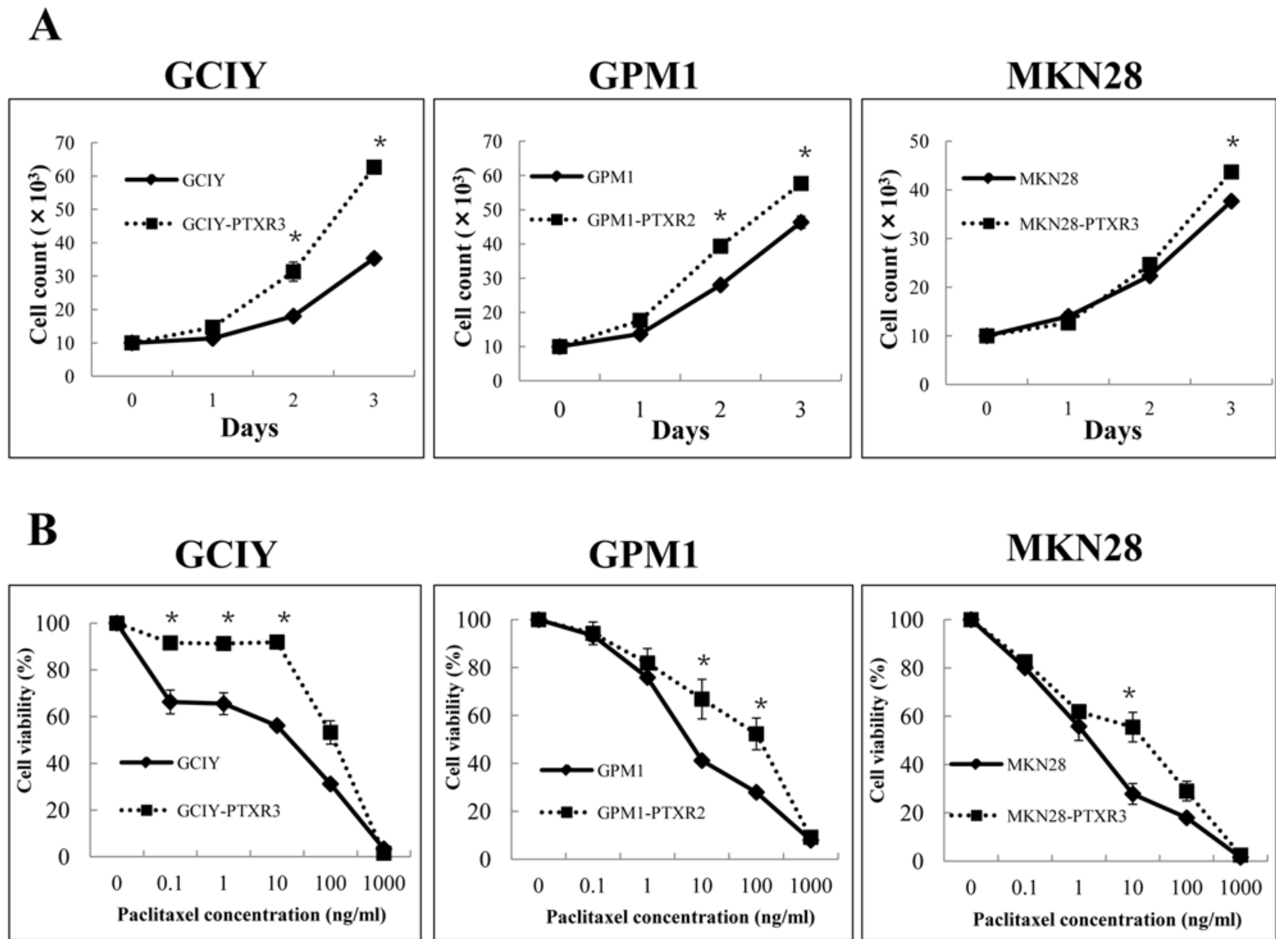

Figure 2. *Comparison of growth characteristics in vitro between parental cells and paclitaxel-resistant cells. Growth curves of parental cells and paclitaxel-resistant cells in vitro (A). Growth inhibition of parental cells and paclitaxel-resistant cells by paclitaxel in vitro (B). Data are average ±SE. *p<0.05.*

greatly in paclitaxel-resistant cells than in parental cells by qRT-PCR analysis. Because there is a gradient for paclitaxel resistance among three cell lines (GCIY>GPM1>MKN28), we further conducted cluster analysis. We found clearly different expression profiling patterns among these three cell lines and selected four genes whose expression ratio (resistant cells/parent cells) correlated with paclitaxel resistance (GCIY>GPM1>MKN28) as follows: *ERBB2IP* (2.27>2.17>0.92), *KIF23* (4.43>1.71>0.98), *ATAD2* (2.95>1.87>1.12) and *PHF19* (2.22>1.73>0.91) (Figure 4C, see A1 and A2).

Validation of selected genes. qRT-PCR analysis of clinical samples confirmed that mRNA expression of the four genes *ERBB2IP*, *KIF23*, *ATAD2* and *PHF19* was significantly higher (p<0.05) in the seven responders than in the five non-responders to paclitaxel, indicating that these four genes would be potential prognostic markers for resistance to paclitaxel (Figure 5).

Discussion

In the present study, we successfully isolated three novel paclitaxel-resistant variant cell lines from parental gastric cancer cell lines. These cell lines are unique for the following reasons. Although paclitaxel-resistant ovarian cancer cell lines such as OM1/Tvivo cells are now available (14, 15), few paclitaxel-resistant gastric cancer cell lines have been reported worldwide (18). Our cell lines, therefore, are the first paclitaxel-resistant gastric cancer cell lines established by the new *in vivo* selection method. To date, ATP-binding cassette transporters (11), β-tubulin isotypes (12) and microRNAs (13) have been reported as candidate genes which are responsible for paclitaxel resistance in

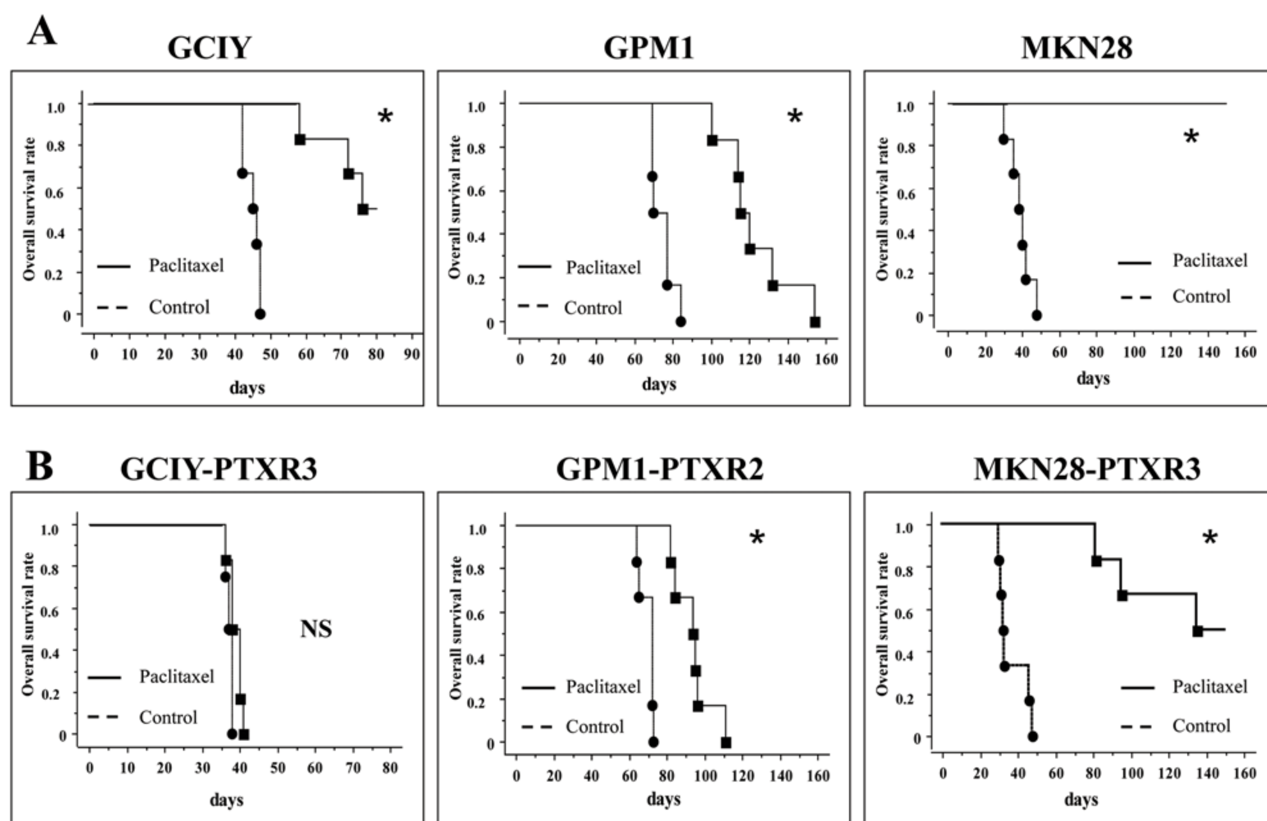

Figure 3. *Overall survival of the intraperitoneally transplanted recipient mice with and without paclitaxel treatment as depicted by the Kaplan-Meier method. Parental cells (A) and paclitaxel-resistant cells (B). NS, not significant; *p<0.05.*

ovarian cancer. In the present study, however, these genes were not up-regulated in our paclitaxel-resistant cell lines, suggesting a difference in the mechanism of resistance between ovarian cancer cells and gastric cancer cells, or between *in vitro*-selected resistant cells and *in vivo*-selected resistant cells. There is a clear gradient for the intensity of paclitaxel resistance among three cell lines in the following order: GCIY>GPM1>MKN28. In fact, MKN28 cells were highly sensitive to paclitaxel and therefore, it was difficult to isolate a paclitaxel-resistant subline from the parental cells until reduction of the usual *i.p.* paclitaxel treatment in mice. In other words, the MKN28 cell line is a good model for paclitaxel-susceptibility. These gastric cancer cell lines would therefore be very useful preclinical models for understanding the detailed mechanism of acquired paclitaxel resistance, as well as for developing new therapies to overcome such resistance in patients with gastric cancer.

Using these paired resistant and parental cell lines as tools, we tried to isolate new genes which are related to paclitaxel resistance by differential gene expression analysis and subsequent hierarchical clustering analysis. Consequently, we successfully selected four candidate genes having a gradient of gene expression related to paclitaxel resistance, namely *KIF23*, *EBB2IP*, *ATAD2* and *PHF19*. These isolated genes can be classified into two categories. The first class includes *KIF23* and *ERBB2IP*. Common features of these proteins are their cell cycle-dependent expression, with maximal expression in the G_2/M phase, and they are closely associated with the mitotic spindles. *KIF23* is a kinesin-like motor protein that localizes to the interzone of mitotic spindles in the nucleus, acting as a plus-end-directed motor enzyme that moves antiparallel microtubules *in vitro* (19). Previous studies have reported that depletion of *KIF23* in HeLa cells induces the formation of multinucleate cells, likely because of a cytokinesis defect (20). These findings indicate the essential role of *KIF23* in cytokinesis and suggest the possibility that overexpression of *KIF23* observed in paclitaxel-resistant cells reinforces G_2/M transition suppressed by paclitaxel, leading to escape from cytokinetic defects. On the other hand, *ERBB2IP* was originally described as a human epidermal growth factor receptor 2(*HER2*)-binding partner. It was known that *ERBB2IP* was constitutively associated with

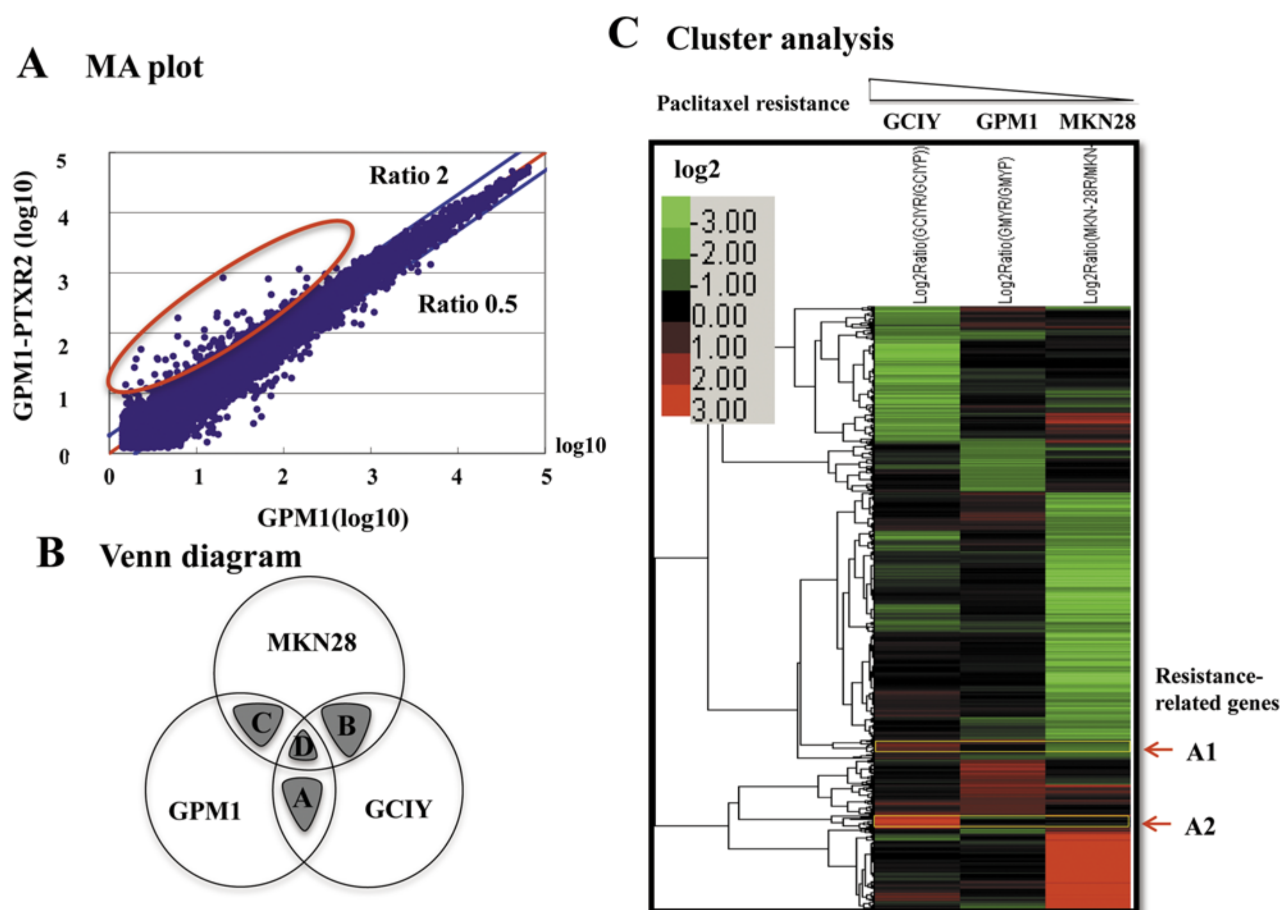

Figure 4. *Gene expression profiling analysis of three pairs of parental and paclitaxel-resistant cell lines. Selection of up-regulated genes in paclitaxel-resistant cell line compared with parental cell line by differential expression analysis using 3D gene oligo 25K chip (A). Venn diagram, showing overlap of up-regulated genes in three paclitaxel-resistant cell lines (B). Selection of up-regulated genes in paclitaxel-resistant cell lines according to the relative intensity of their paclitaxel-resistance by cluster analysis. The log2 ratio of paclitaxel-resistant cells/parental cells in each cell lines is shown with a labeled bracket. Red, black, and green indicate a fold-change expression level (C).*

HER2 receptor and directly bound to the C-terminus of *HER2*, guiding the basolateral localization of *HER2*. Recently, however, Liu *et al*. reported that in addition to basolateral membrane localization, *ERBB2IP* is exceptionally located in the nucleus in the mitotic cells with remarkable increase at the G_2/M phase. They further demonstrated that inactivation of *ERBB2IP* causes an acceleration of the G_1/S transition, the formation of multipolar spindles and abnormal chromosome congression (21). These results suggest the possibility that the overexpression of *ERBB2IP* seen in paclitaxel-resistant cells normalizes spindle formation and allows cells to escape from mitotic defect.

The second class of candidate genes for paclitaxel-resistance are *ATAD2* and *PHF19*, both of which were recently found to be involved in the epigenetic control of gene expression. *ATAD2* (ANCCA) possesses an AAA-type ATPase domain and a bromodomain that recognize specifically acetylated histones such as H3K14ac (22) and regulate expression of genes such as androgen receptor(*AR*) and estrogen receptor α(*ERα*). Another gene, *PHF19* is a member of the polycomb-like family which binds trimethylated histone H3 Lys36 (H3K36me3) as a repressive chromatin modifier and is known to be involved in the epigenetic control of gene expression (23). DNA methylation and histone acetylation status of several genes in ovarian cancer such as ATP-binding cassette sub-family G member 2 (*ABCG2*) and Enhancer of zeste homolog 2(*EZH2*) have been reported to be associated with drug resistance (24). However, at present, the relationship between these two genes and paclitaxel resistance remains totally unknown and warrants further studies.

In conclusion, we developed three new *i.p.* paclitaxel-resistant gastric cancer cell lines and extracted four candidate

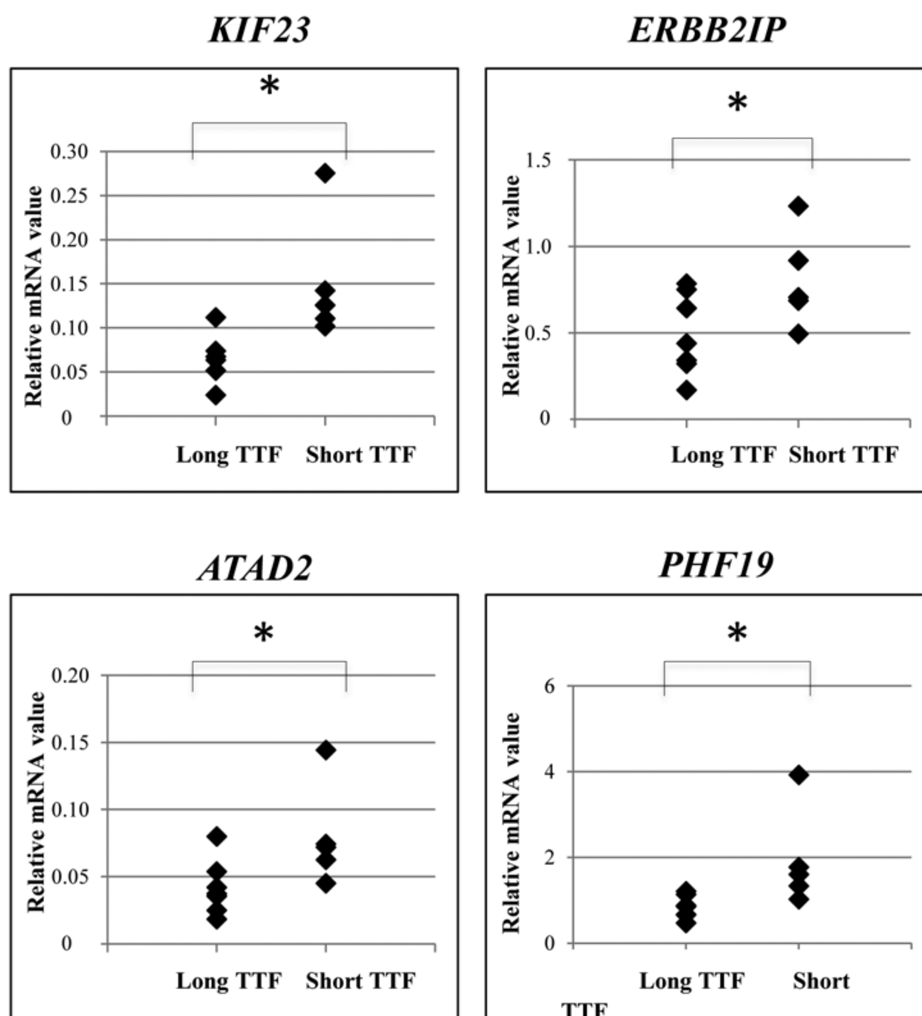

Figure 5. *Validation of candidate genes using clinical specimens from responder and non-responders to paclitaxel. Expression of candidate genes of surgically resected tumor tissues freshly isolated from patients with long time to treatment failure (TTF) (responder, n=7) and short TTF (non-responder, n=5) was examined by qRT-PCR. Relative mRNA value in the vertical axis is represented as a specific gene mRNA/GAPDH mRNA ratio. *p<0.05.*

genes for paclitaxel resistance of gastric cancer, to our knowledge, for the first time. These cell lines would be excellent preclinical models for understanding the mechanism of paclitaxel resistance and for developing a new therapy for patients with paclitaxel-resistant gastric cancer. Although the precise role of these candidate genes in paclitaxel resistance still remains unclear, these genes would be good predictive makers for *i.p.* paclitaxel resistance of gastric cancer cells. Further study is needed to clarify the role of these genes in paclitaxel resistance.

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