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

HIROKI MURAKAMI1,3, SEIJI ITO2, HARUNARI TANAKA1, EISAKU KONDO1, YASUHIRO KODERA3 and HAYAO NAKANISHI1

1Division of Oncological Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan; 2Department of Gastroenterological Surgery, Aichi Cancer Center Central Hospital, Nagoya, Japan; 3Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Nagoya, Japan

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 an 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.

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 antibodies is one potential alternative for conventional chemotherapy. To date, however, only a few pre-clinical studies and clinical trials for peritoneal metastasis of gastric cancer have been reported (5).

Paclitaxel is a mitotic inhibitor that binds β-tubulin and thereby stabilizes microtubules, interferes with breakdown of microtubules and consequently inhibits the progression of the G2/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 pre-clinical models (6). Intraperitoneal 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 do 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 an ovarian cancer cell line established in vitro selection method exhibited high paclitaxel resistance in in vitro culture conditions, but lost this resistance or tumorigenicity in a transplanted tumor model in mice (15), indicating some difficulty in establishing a clinically-suited paclitaxel-resistant model by the in vitro selection method. Furthermore, in gastric cancer, there are 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 analyses 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). GPM1 cell line was established previously in our laboratory (Aichi Cancer Center Research Institute, Japan) (16). GCIY and GPM1 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 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 mRNA expression arrays were then hybridized, and subsequently scanned with a DNA microarray scanner (3D-Gene Scanner; Toray Industries, Inc., Tokyo, Japan). 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. Red, black, and green indicate a fold-change expression level above, at, and below 1, respectively.

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 MessageAmpTM 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. 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 mRNA expression arrays were then hybridized, and subsequently scanned with a DNA microarray scanner (3D-Gene Scanner; Toray Industries, Inc., Tokyo, Japan). 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. Red, black, and green indicate a fold-change expression level above, at, and below 1, respectively.
transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA). 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 used 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 (Table I) are as follows: KIF23 sense primer 5'-GCCGAGAGCTACAGGTC-3', antisense primer 5'-GACCGGGTGACATTTCA-3'; probe no. 80; ERBB2IP sense primer 5'-CTCTTGTTGAGATTTCCAAACG-3', antisense primer 5'-TGGGTGATGCTGTAATTT-3', probe no. 71; ATAD2 sense primer 5'-GCCGAGAGCTACAGGTC-3', antisense primer 5'-GACCGGGTGACATTTCA-3', probe no. 80; PHF19 sense primer 5'-AAAGTGTGGCTGCCAAGAAC-3', antisense primer 5'-GCTTGTCAGCTTGGTGTT-3', probe no. 55; ATAD2 sense primer 5'-GCCGAGAGCTACAGGTC-3', antisense primer 5'-GACCGGGTGACATTTCA-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 (II/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 intraperitoneally. 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.

Results

Establishment of i.p. paclitaxel-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 lower 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 a 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 mouse-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, survival benefits

---

**Table 1. List of candidate genes selected for i.p. paclitaxel resistance in this study**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene names</th>
<th>Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIF23</td>
<td>Kinesin family member-23</td>
<td>A kinesin-like motor protein that localizes to the interzone of mitotic spindles in the nucleus composed by microtubules</td>
</tr>
<tr>
<td>ERBB2IP</td>
<td>Erbb2 interacting protein</td>
<td>It binds to the ERBB2 protein and regulates ERBB2 function and membrane localization.</td>
</tr>
<tr>
<td>ATAD2</td>
<td>ATPase family, AAA domain containing-2</td>
<td>It also localizes in the nucleus of the mitotic cells with increase at G2/M phase</td>
</tr>
<tr>
<td>PHF19</td>
<td>PHD finger protein-19</td>
<td>AAA-type ATPase domain that recognize specifically acetylated histones such as H3K14ac</td>
</tr>
</tbody>
</table>

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.

---

Murakami et al: Paclitaxel Resistance-related Genes in Gastric Cancer
obtained from *i.p.* paclitaxel treatment in paclitaxel-resistant cells were 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 two-fold 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 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 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 could 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 OMI/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), ş-tubulin isotypes (12) and microRNAs (13) have been reported as candidate genes which are responsible for paclitaxel resistance in 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 sub-line 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 pre-clinical 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 EBB2IP. Common features of these proteins are their cell cycle-dependent expression, with maximal expression in the G2/M phase, and they are closely associated with the mitotic spindles. KIF23 is a kinesin-like motor protein that localizes to the inter-zone of mitotic spindles in the nucleus, acting as a plus-end-directed motor enzyme that moves anti-parallel 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 G2/M transition suppressed by paclitaxel, leading to escape from cytokinetic defects. On the other hand, EBB2IP was originally described as a human epidermal growth factor receptor-2 (HER2)-binding partner. It was known that EBB2IP was constitutively associated with 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

Figure 3. Overall survival of 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.
membrane localization, ERBB2IP is exceptionally located in the nucleus in the mitotic cells with remarkable increase at the G2/M phase. They further demonstrated that inactivation of ERBB2IP causes an acceleration of the G1/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 tri-methylated 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 genes for paclitaxel resistance of gastric cancer, to our knowledge, for the first time. These cell lines would be excellent pre-clinical models for understanding the

Figure 4. Gene expression profiling analysis of three pairs of parental and paclitaxel-resistant cell lines. Selection of up-regulated genes in a 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 line is shown with a labeled bracket. Red, black, and green indicate a fold-change expression level (C).
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.

Acknowledgements

We thank Mrs. M. Yoshimura and K. Nishida for expert technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by a Grant-in-Aid for Priority Research Project from Knowledge Hub Aichi, Japan.

References


Received August 14, 2013
Revised September 18, 2013
Accepted September 19, 2013

Murakami et al: Paclitaxel Resistance-related Genes in Gastric Cancer

4307