Original Article

P-glycoprotein plays an important role in the cross-resistance to taxanes in 5FU-resistant gastric cancer cells

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Background: 5FU is a key drug used in chemotherapy regimens for advanced and metastatic gastric cancer, and is often used as a first-line therapy. Taxanes, such as paclitaxel and docetaxel, are newer chemotherapeutic agents that are usually used as second-line therapies. However, second-line chemotherapy is sometimes not as effective as expected, even when the second-line agents have different mechanisms of action from those of the first-line agents. Therefore, it is important to understand the mechanisms of cross-resistance in order to provide more efficacious treatment.

Methods: The present study compared the characteristics of 5FU-resistant MKN45/F2R cells and parental MKN45 cells in order to clarify the mechanisms of cross-resistance between 5FU and taxanes.

Results: The MKN45/F2R cells showed resistance to 5FU, paclitaxel and docetaxel (IC50: 82.3 μM, 254.9 nM, 27.0 nM, respectively) in comparison to the MKN45 cells (IC50: 1.05 μM, 0.28 nM, 5.20 nM, respectively). We then examined the changes in the expression levels of various molecules related to 5FU resistance, including thymidylate synthase (TS), orotate phosphoribosyltransferase (OPRT) and dihydropyrimidine dehydrogenase (DPD). The ternary complex of TS emerged only after treatment with MKN45/F2R using a 100-fold concentration of 5FU, and required a 6-fold longer time to form in comparison to that in the MKN45 cells. Meanwhile, the expression of OPRT was decreased to 42% in the MKN45/F2R cells, while the MKN45 cells showed a 62.5-fold increase in resistance following transfection with siRNA against OPRT. These findings strongly indicate that a decrease in OPRT plays an important role in the onset of resistance to 5FU. However, OPRT knockdown did not contribute to resistance to paclitaxel or docetaxel. We therefore also examined the expression levels of several molecules related to taxane resistance, including p-glycoprotein, β-III tubulin and Bcl-2, and found an increased expression of p-glycoprotein in the MKN45/F2R cells compared with the parental MKN45 cells (4.5-fold). Furthermore, the inhibition of p-glycoprotein by verapamil reversed the change in the IC50 for paclitaxel (0.216±0.0416 nM) and docetaxel (5.64±0.442 nM) in the MKN45/F2R cells to a level similar to that noted in the parental MKN45 cells (0.368±0.101, 5.21±0.603 nM, respectively).

Conclusions: These findings suggest that p-glycoprotein plays a critical role in the development of cross-resistance to taxanes in 5FU-resistant cells. Interestingly, p-glycoprotein cannot transport 5FU, and it remains unclear why 5FU-resistant cells express p-glycoprotein. However, our results may suggest that taxanes should be used prior to 5FU, as 5FU is considered to be effective despite the increased expression of p-glycoprotein in tumor cells.

Keywords: Fluorouracil; paclitaxel; docetaxel; p-glycoprotein

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Introduction

In spite of recent advances in the development of adjuvant therapy (1) and diagnostic tools, recurrent tumors are often detected even after curative surgery, and many patients with gastric cancer are still diagnosed only at the late stages after surgery is no longer curative. Although the median survival (MST) of metastatic gastric cancer improved from 3-5 to 13 months following the development of new chemotherapeutic agents including S-1, taxanes, CPT-11 and platinum derivatives (2-5), gastric cancer remains one of the major causes of cancer death worldwide (6). Most patients with advanced and metastatic gastric cancer are treated with multiple-line chemotherapies, and sometimes, second-line chemotherapy is not as effective as expected even when the second-line agents have different mechanisms of action from the first-line agents (7,8).

Many different chemotherapeutic regimens used for gastric cancer and gastrointestinal malignancies include 5FU (1,2,9). The first step in the activation of 5FU is the phosphorylation of 5FU by orotate phosphoribosyltransferase (OPRT), which metabolizes 5FU to 5-fluorouridine monophosphate (FUMP) in the presence of 5-phosphoribosyl 1-pyrophosphate. Then, 5FU is finally metabolized to its active metabolite, 5-fluorodeoxyuridine diphosphate (FdUMP), and it forms a covalent ternary complex with the DNA *de novo* synthesizing enzyme, thymidine synthetase (TS), together with the coenzyme, 5,10-methylenethtrahydrofolate (MTHF). This complex blocks the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP) and thus inhibits DNA synthesis. 5FU is catabolized to 2-fluorob-alanin by dihydropyrimidine dehydrogenase (DPD) (10). Therefore, the enzymes involved in the metabolism of 5FU, such as OPRT, TS and DPD, could be predictive markers for the response to 5FU (10).

Taxanes, such as docetaxel and paclitaxel, are novel chemotherapeutic agents that promote the assembly of microtubules from tubulin dimers, and inhibit the depolymerization of tubulin, which stabilize microtubules in the cell. This results in the inhibition of DNA, RNA and protein synthesis (11). Docetaxel and paclitaxel have shown promising activity in gastric cancer, both as monotherapy (12) and in combination with other agents (3,4,13).

MKN45/F2R cells with reduced OPRT gene expression in comparison to MKN45 parent cells were previously established in order to elucidate the mechanism of 5FU resistance (14). Interestingly, the MKN45/F2R cells also showed resistance to docetaxel and paclitaxel, as well as other chemotherapeutic agents.

It is important to understand the mechanism of cross-resistances for more efficacious treatment. The present study was performed to clarify the mechanism of cross-resistance between 5FU and taxanes in 5FU-resistant cells, and also discusses a novel strategy to overcome such cross-resistance.

Materials and methods

Drugs

The 5FU, paclitaxel, docetaxel were kindly provided by Kyowa Hakko (Tokyo, Japan), Bristol-Myers Squibb (Tokyo, Japan), Sanofi Aventis (Tokyo, Japan), respectively. Verapamil was purchased from Wako (Osaka, Japan).

Cell lines and cell culture

MKN45 cells are poorly differentiated human gastric adenocarcinoma cells. The MKN45 cells were cultured in RPMI 1640 medium (Wako) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St., Louis, MO, USA), antibiotics (Sigma-Aldrich), and HEPES (Sigma-Aldrich) in a humidified atmosphere of 5% CO_2 at 37 ℃. MKN45/F2R cells are a 5FU-resistant cell line. This line was established by continuously exposing the MKN45 parent cells to increasing concentrations (0.1-2 μM) of 5FU over the course of a year. The MKN45/F2R cells were routinely maintained in culture medium containing 2 μM of 5FU. The resistant cells were cultured in drug-free medium for at least 2 weeks before all of the studies to eliminate the effects of 5FU in the experiments (14).

Western blot analysis and antibodies

The cells were harvested and lysed in RIPA buffer (Sigma-Aldrich) for 15 minutes on ice. The protein concentration of the lysates was measured using a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The cell lysates were boiled in Sample Buffer Solution (Wako). Total cell protein extracts (20 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using SuperSep™ (Wako), and were electrophoretically transferred onto polyvinyl difluoride membranes. The membranes were blocked with PVDF blocking reagent (TOYOBO, Osaka,

Japan) for 1 h. The membranes were then incubated with primary antibodies against β-actin, DPD (purchased from Cell Signaling Technology 1:5,000), TS and OPRT (kindly provided by Taiho Pharmaceutical Company, Tokyo, Japan 1:10,000) overnight at 4 ℃. The primary antibodies were diluted with Can Get Signal Solution 1 (TOYOBO). The membranes were then washed with Dako Washing Buffer (Dako, Denmark) and incubated with the appropriate secondary antibodies (Millipore 1:25,000). The secondary antibodies were diluted with Can Get Signal Solution 2 (TOYOBO). The immunoreactive proteins were visualized by chemiluminescence using ImmunoStar LD reagents (Wako), and images were captured by a LAS-4000 device (FUJIFILM, Tokyo, Japan).

The 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay for the effects of 5FU or oxaliplatin

Cell growth was assessed by a standard 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay, which detects the dehydrogenase activity in viable cells. A total of 5×10^3 cells were seeded onto each well of 96-well culture plates, and were cultured for 24 h. The cells were treated with various concentrations of drugs for 72 h, the culture medium was removed, and 100 μL of a 0.5 mg/mL solution of MTT (Sigma-Aldrich) was added to each well. The plates were then incubated for 4 h at 37 ℃. The culture medium was replaced with 100 μL of dimethyl sulfoxide (Wako) per well, and the absorbance at 540 nm was measured using an Envision 2104 Multilabel Reader (Perkin Elmer, Waltham, MA, USA). Each assay was repeated three times, and the mean half maximal inhibitory concentration (IC50) values were calculated based on the results of the MTT assay. The significance of differences in IC50s was tested using Student's *t*-test.

Transfection and small interfering RNA experiments for OPRT

The MKN45 cells were cultured in medium without antibiotics for 24 h to 50-70% confluence before transfection. The cells were transfected with a small interfering RNA (siRNA) oligonucleotide using Lipofectamine RNAiMAX (Invitrogen) in a final siRNA concentration of 40 nmol/L in serum-free Opti-MEM (Invitrogen) for 48 h. The total RNA and proteins were extracted, and the expression levels of the OPRT mRNA and protein were analyzed by real-time RT-PCR and a Western blotting analysis, respectively. The siRNA oligonucleotides for OPRT (Stealth RNAi) and the negative control oligonucleotides (Stealth RNAi siRNA Negative Control) were purchased from Invitrogen.

Results

Changes in the expression levels of the TS, DPD and OPRT

The cells used in the present study were parental MKN45 cells and 5FU-resistant MKN45/F2R cells that were previously established as 5FU-resistant cells. MKN45/F2R cells showed 78.3-fold increased resistance to 5FU in comparison to the parental MKN45 cells. The MKN45/F2R cells also showed resistance to paclitaxel and docetaxel. The major characteristics of these cell lines were consistent with those reported previously (14).

TS, DPD and OPRT play key roles in the functions of 5FU. Therefore, the expression of the proteins was examined to understand the mechanism of acquired resistance in the MKN45/F2R cells.

Both cell lines were first treated with 1, 10 and 100 μM 5FU for 24 h, and the expression levels of the TS, DPD and OPRT proteins were investigated by a Western blot analysis. *Figure 1A* shows that the expression of TS was detected as double bands. The lower band (smaller molecular weight) was considered to be free TS (36kD), and the upper band (larger molecular weight) was considered to be the ternary complex formed by TS, FdUMP and MTHF. The ternary complex in the parental MKN45 cells emerged when they were treated with 1 μM 5FU. The band for the ternary complex was markedly reduced by treatment with 1 and 10 μM in the 5FU-resistant MKN45/F2R cells. The ternary complex emerged in both cell types following treatment with 100 μM of 5FU (*Figure 1A*). Both cell types were then treated with 10 μM of 5FU for 3, 6, 12, 24 and 48 h. The ternary complex in MKN45 cells emerged after 3 h, while it emerged only after 24 h in the MKN45/F2R cells (*Figure 1B*).

The expression of OPRT was decreased to 42% in the MKN45/F2R cell line in comparison to the MKN45 parental cell line, and was not altered by treatment with 5FU in either cell type (*Figure 1A,B*). DPD was detected in both cell lines, and there was no significant difference in the expression between the two cell lines (*Figure 1A,B*).

These results indicated that the decreased expression of OPRT led to reduced formation of the TS ternary complex.

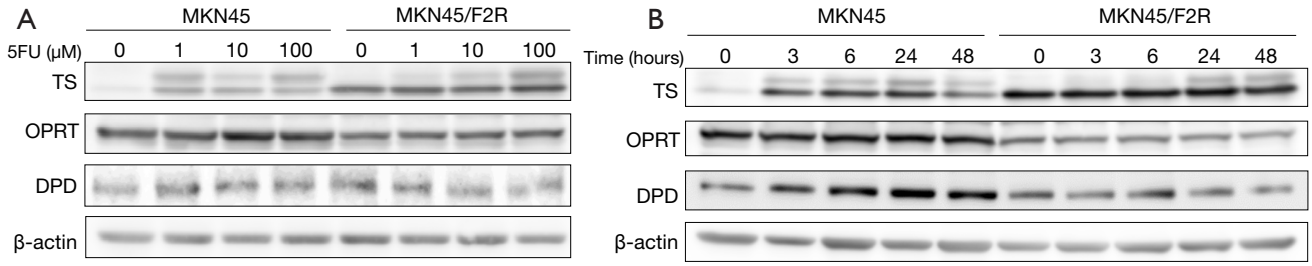

Figure 1 The changes in TS, DPD and OPRT expression after 5FU treatment in MKN45 and MKN45/F2R cells. The results of a Western blot analysis for TS, DPD and OPRT was performed after 5FU treatment. (A) MKN-45 and MKN-45/F2R cells were treated with 5FU at concentrations of 1, 10 and 100 μM for 24 h; (B) the MKN-45 and MKN-45/F2Rc ells were treated with 10 μM of 5FU for 3, 12, 24 and 48 h. TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; OPRT, orotate phosphoribosyltransferase.

Therefore, decreased OPRT may be one of the causes of 5FU resistance in MKN45/F2R cells.

Increased sensitivity to 5FU, and unchanged sensitivity to taxanes, after transfection of a siRNA against OPRT in MKN45 cells

A siRNA against OPRT was transfected into the MKN45 parental cells, and the sensitivity was analyzed to confirm whether the decreased expression of OPRT directly induced resistance to 5FU or taxanes. The mRNA and protein expression levels of OPRT in the transfected cells were investigated before analyzing the IC50, and they were found to be markedly decreased to 10.3% and 50.0%, respectively, in untreated cells (*Figure 2A*,*B*). Next, the IC50 values for 5FU, paclitaxel and docetaxel were examined by using an MTT assay. The IC50 values for 5FU in the MKN45 cells transfected with siRNA increased to 65.6 μM, thus 62.5-fold resistance was obtained after transfection (*Figure 2C*, *Table 1*). Meanwhile, the resistance to paclitaxel and docetaxel in the MKN45 cells was not altered after siRNA transfection (*Figure 2D*,*E*). The IC50 values under these conditions are shown in *Table 1*.

Increased p-glycoprotein expression contributes to cross-resistance to taxanes in 5FU-resistant MKN45/F2R cells

The expression of several molecules was examined using a Western blot analysis to elucidate the mechanism underlying the cross-resistance to taxanes in the MKN45/F2R cells. The expression levels of p-glycoprotein, β-III tubulin and Bcl-2, which are involved in taxane resistance, were examined in the parental MKN45 cells and 5FU-resistant MKN45/F2R cells. The expression of p-glycoprotein was increased 4.5-fold in the MKN45/F2R cells in comparison to the MKN45 parental cells (*Figure 3A*). P-glycoprotein is an efflux pump for taxanes and other drugs, such as doxorubicin (15), and it can be competitively inhibited by verapamil (16). The sensitivity of the cells to 5FU and taxanes was analyzed in the presence and absence of verapamil to confirm whether p-glycoprotein contributes to the resistance to 5FU or taxanes.

The cells were cultured in media containing various concentrations of paclitaxel, docetaxel or 5FU with or without 10 μM of verapamil, and the cell viability was measured using the MTT assay. *Figure 3B* shows that the resistance to 5FU in the MKN45/F2R cells was not reversed when they were cultured with verapamil. Meanwhile, the resistance to paclitaxel and docetaxel was reversed to the level of the parental MKN45 cells when they were cultured with verapamil (*Figure 3C*,*D*). The IC50 values under these conditions are shown in *Table 2*. These results indicated that p-glycoprotein played a principal role in the cross-resistance to taxanes, but it did not contribute to 5FU resistance in MKN45/F2R cells.

Discussion

The roles of TS, DPD and OPRT in 5FU metabolism have been studied by many researchers. OPRT has received extensive attention because its expression is believed to be correlated with the sensitivity to 5FU (17,18). The sensitivity to 5FU was increased in OPRT transfected cells *in vitro* and *in vivo* (19). The 5FU-resistant MKN45/F2R cells were established from MKN45 gastric cancer cells to study the mechanism underlying drug resistance in gastric cancer (14). The MKN45/F2R cells showed 78.3-fold increased resistance to 5FU compared to the parental cells,

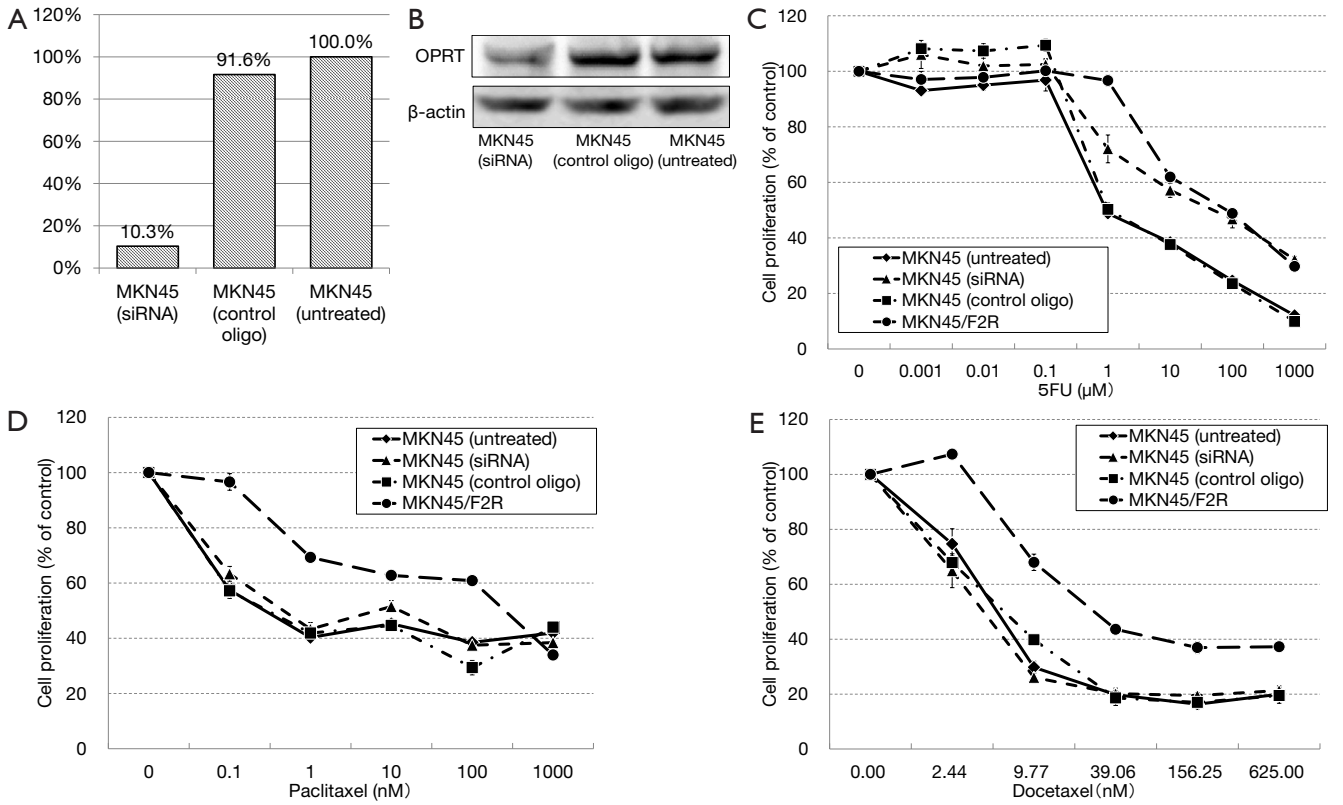

Figure 2 The effects of siRNA-mediated OPRT knockdown in MKN45 cells. A siRNA against OPRT and negative control were transfected into parental MKN45 cells, and the IC50s for 5FU, paclitaxel and docetaxel in the MKN45, MKN45 (negative control), MKN45 (siRNA) and MKN45/F2R cells were analyzed by the MTT assay. (A) The mRNA levels of OPRT after siRNA transfection; (B) the results of a Western blot analysis of the OPRT expression after siRNA transfection; (C) the results of the MTT assay for 5FU in these cells; (D) the results of the MTT assay for paclitaxel in these cells; (E) the results of the MTT assay for docetaxel in these cells. OPRT, orotate phosphoribosyltransferase; MTT, 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide.

Table 1 The IC50 values for 5FU and taxanes in the MKN45, MKN45/F2R and MKN45 cells transfected with siRNA against OPRT

Drugs	MKN45	MKN45 (siRNA)	MKN45/F2R
5FU (μM)	1.05±0.178	65.6±3.85*	82.3±8.87*
Paclitaxel (nM)	0.28±0.0654	1.02±0.606	254.9±8.92*
Docetaxel (nM)	5.20±0.602	4.15±0.689	27.0±1.39*

MKN45 cells were transfected with a siRNA against OPRT, and MTT assays were performed three times. The resulting IC50 values were compared to those of the parental MKN45 cells and 5FU-resistant MKN45/F2R cells. *, P<0.01 based on Student's *t*-test, compared to the IC50 value in parental MKN45 cells. OPRT, orotate phosphoribosyltransferase; MTT, 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide; IC50, half maximal inhibitory concentration.

and the expression of OPRT was decreased in these cells. Furthermore, OPRT-knockdown MKN45 cells showed resistance to 5FU (62.5-fold) in comparison to the control cells. These results strongly indicated that decreased OPRT expression plays an important role in the resistance to 5FU in gastric cancer cells.

Tumor cells that are resistant to one anticancer drug often acquire resistance to other drugs, and the MKN45/F2R cells also showed cross-resistance to paclitaxel and docetaxel, as well as other drugs. P-glycoprotein, β3 tubulin and Bcl-2 are associated with resistance to taxanes (20-22), and the expression of p-glycoprotein in the 5FU-resistant

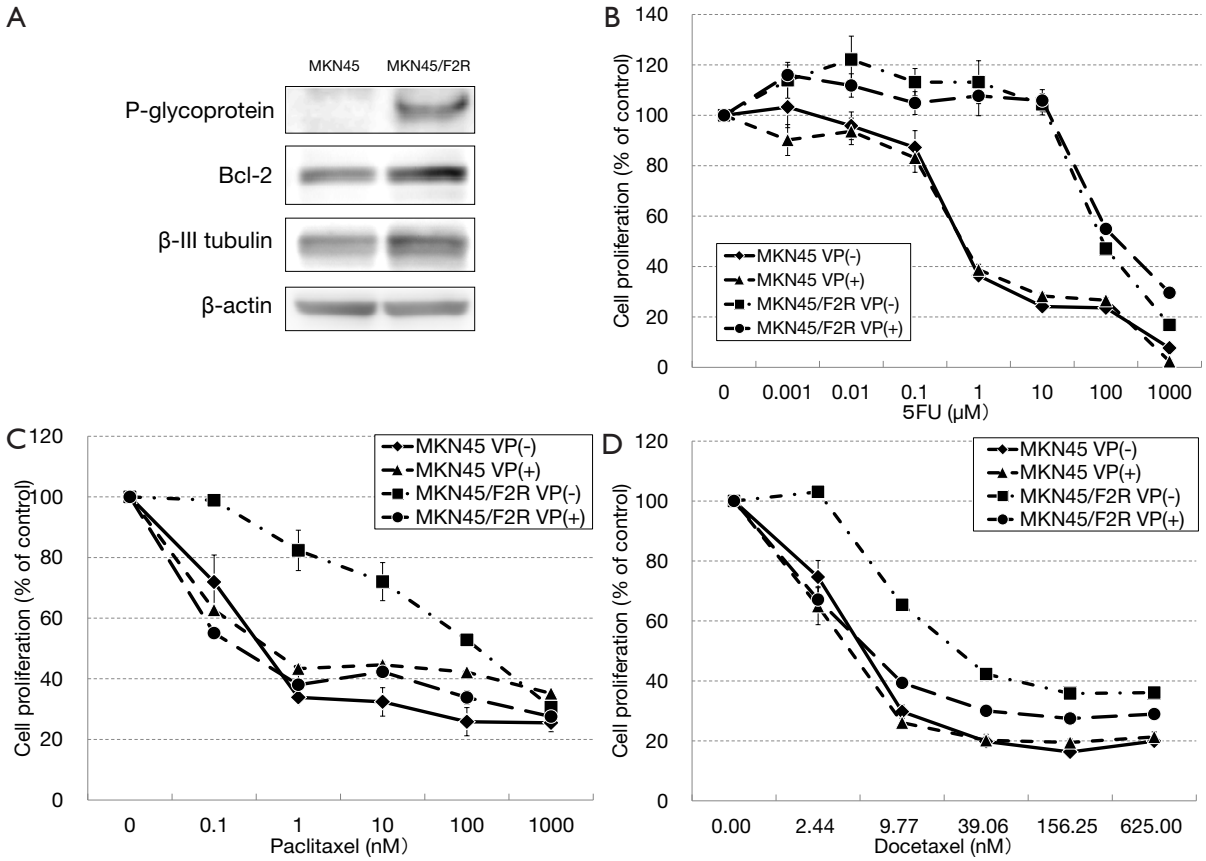

Figure 3 The results of a Western blot analysis of the proteins related to taxane resistance, and the MTT assay in the presence of verapamil. Western blotting for p-glycoprotein, Bcl-2 and β-III tubulin was performed, and the expression levels were compared in MKN45 and MKN45/F2R cells. MTT assays for 5FU, paclitaxel and docetaxel were performed in the presence of VP to inhibit p-glycoprotein. (A) The results of a Western blot analysis of the expression of p-glycoprotein, Bcl-2 and β-III tubulin; (B) the results of the MTT assay for 5FU with or without VP in both cell lines; (C) the MTT assay for paclitaxel with or without VP; (D) the results of the MTT assay for docetaxel with or without VP. VP, verapamil; MTT, 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide.

Table 2 The IC50 values for 5FU and taxanes with or without verapamil treatment in MKN45 and MKN45/F2R cells

Drug(s)	MKN45	MKN45/F2R	Fold-change
5FU	0.535±0.0717 (μM)	89.7±5.87 (μM)	167
5FU + verapamil	0.560±0.0840 (μM)	141.6±8.33 (μM)	252
Paclitaxel	0.368±0.101 (nM)	133.3±17.4 (nM)	362
Paclitaxel + verapamil	0.463±0.0450 (nM)	0.216±0.0416 (nM)*	0.467
Docetaxel	5.21±0.603 (nM)	24.5±0.757 (nM)	4.70
Docetaxel + verapamil	4.12±0.690 (nM)	5.64±0.442 (nM)*	1.37

Verapamil was added to the media with 5FU, paclitaxel or docetaxel, and the IC50 values were analyzed by an MTT assay. The resistance to 5FU was not decreased by adding verapamil in the MKN45/F2R cells. However, the resistance to both paclitaxel and docetaxel was reversed by verapamil. *, P<0.01 based on Student's *t*-test, compared to the IC50 value for the same drug without verapamil in the same cell line. MTT, 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide; IC50, half maximal inhibitory concentration.

MKN45/F2R cells was markedly increased in comparison to parental MKN45 cells. P-glycoprotein is an ATP-dependent drug efflux pump for drugs such as taxanes and doxorubicin (15), and it can be competitively inhibited by verapamil (16). The resistance to docetaxel and paclitaxel in MKN45/F2R were reversed in the presence of verapamil, suggesting that the increased expression of p-glycoprotein was the main cause of the taxane resistances in 5FU-resistant MKN45/F2R cells.

These results raised another question. P-glycoprotein can transport various hydrophobic agents, such as taxanes and doxorubicin, but 5FU is hydrophilic, and cannot be transported by p-glycoprotein (23). The resistance to 5FU in MKN45/F2R cells was not reversed in the presence of verapamil. Therefore, it is unclear why 5FU-resistant MKN45/F2R cells expressed p-glycoprotein.

However, these results do offer a suggestion. Constant exposure to 5FU may lead to elevated expression of p-glycoprotein, causing cancer cells develop cross-resistance to taxanes (24). Some researchers have reported that 5FU is still effective even when increased expression of p-glycoprotein was recognized in tumor cells (23,25,26). These findings may suggest that taxanes should be used prior to 5FU. This is contradicts the standard strategy for first-line therapy. The exposure to taxanes may lead to elevated expression of p-glycoprotein in cancer cells, but 5FU may still be effective even after treatment with taxanes because it is not subject to p-glycoprotein-mediated transport (23,25). Although the present study did not clarify whether exposure to taxanes induced the expression of p-glycoprotein, but this has been noted in other studies (23,25).

In conclusion, the current study revealed that p-glycoprotein plays a principal role in the cross-resistance to taxanes in 5FU-resistant gastric cancer cells, and suggests that treatment with taxanes prior to 5FU should be considered for patients with gastric cancer.

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Authors' Contributions: R Mori designed the overall study, carried out experiments, collected and analyzed data, and wrote the paper. K Yoshida supervised this study, designed experiments and edited the paper. N Okumura, K Yamaguchi, and M Futamura advised R Mori on interpretation of data, and reviewed the paper. T Tanahashi, K Yawata, and J Kato designed and carried out the experiments. All authors read and approved the final manuscript.

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