Decreased orotate phosphoribosyltransferase activity produces 5-fluorouracil resistance in a human gastric cancer cell line

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Abstract. To elucidate the mechanism of resistance to 5-fluorouracil (5-FU) in human gastric cancer cells, we established a cell line MKN45/F2R, which acquired 5-FU resistance as a result of continuous exposure to increasing dosages of 5-FU over a year. The cell line showed 157-fold elevated 5-FU resistance compared to the MKN45 human gastric cancer parental cell line. Furthermore, the cells acquired crossresistance to paclitaxel and docetaxel. To identify the mechanism of 5-FU resistance, the expressions of 5-FU metabolic enzymes were examined. Although protein expression and activity of thymidylate synthase and dihydropyrimidine dehydrogenase did not change, orotate phosphoribosyltransferase (OPRT) protein expression and activity significantly decreased in the 5-FU resistant MKN45/F2R cells. Interestingly, expression of proteins related to taxane resistance including P-glycoprotein, class III ß-tubulin and Bcl-2 increased in MKN45/F2R cells. OPRT-knockout MKN45 parent cells using small interfering RNA demonstrated 15.8-fold increased resistance to 5-FU compared to the control cells. However, resistance to paclitaxel and docetaxel was not observed. These results strongly indicate that decreased activity of OPRT plays an important role in the acquired resistance of gastric cancer cells towards 5-FU; however, it does not play a direct role in paclitaxel and docetaxel resistance. Further studies are now underway to identify genes related to crossresistance to these chemotherapeutic agents.

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Introduction

Molecular analyses regarding the prediction of the response and/or adverse effects of chemotherapeutic agents aim for personalized treatment of cancer patients, as we have recently explored in microarray analysis studies and clinical trials (1-3). For these purposes, identification of molecular markers for chemotherapeutic agents is critically important. Of the various chemotherapeutic agents, 5-fluorouracil (5-FU) is widely used in a variety of tumors including colorectal, stomach, and breast cancer (4-6). Moreover, the metabolism of 5-FU is well-known.

5-FU is thought to act through its active metabolite 5-fluorodeoxyuridine diphosphate, which, along with coenzyme 5,10-methylenetetrahydrofolate, forms a covalent ternary complex with the DNA *de novo* synthesizing enzyme thymidylate synthase (TS), blocking the conversion of deoxyuridine monophosphate and thus, inhibiting DNA synthesis. Although the cytotoxic effects of 5-FU are directly mediated via the anabolic pathway, 80% of the 5-FU administered is catabolized by the enzyme dihydropyrimidine dehydrogenase (DPD) (7,8). Pharmacogenetic variability of these enzymes might be a major determinant of variations in the outcome of cancer patients treated with 5-FU (9-14). In the anabolic pathway of 5-FU, the first step in the activation of the drug is phosphorylation of 5-FU by orotate phosphoribosyltransferase (OPRT), which directly metabolizes 5-FU to 5-fluorouridine monophosphate (FUMP) in the presence of 5-phosphoribosyl 1-pyrophosphate. We have recently reported that enhanced activation of OPRT is correlated with an enhanced response to 5-FU in cancer patients (15-17), and the enzymes involved in the metabolism could be predictive markers for the response of 5-FU.

Conversely, a significant number of tumors often fail to respond to chemotherapy because they are less sensitive or develop resistance to anticancer drugs after consecutive treatments. Therefore, it is extremely important to understand the mechanism of resistance to chemotherapy for more efficacious treatment of tumors with anticancer drugs. To clarify the mechanism of resistance to 5-FU, we have established cell line MKN45/F2R, which acquired the

resistance to 5-FU, and analyzed the precise mechanism of the phenomenon.

Materials and methods

Drugs. 5-FU, cisplatin, and paclitaxel were purchased from Kyowa Hakko (Tokyo, Japan), Nippon Kayaku (Tokyo, Japan), and Bristol-Myers Squibb (Tokyo, Japan), respectively. SN38 and oxaliplatin were purchased from Yakult Honsha (Tokyo, Japan). Docetaxel was kindly provided by Sanofi Aventis (Tokyo, Japan).

Cell lines and cell culture. MKN45, which are poorly differentiated human gastric adenocarcinoma cells, were cultured in RPMI-1640 medium (Nissui, Tokyo, Japan) with 5% fetal bovine serum (Whittaker MA Bioproducts, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 at 37˚C. To establish a 5-FU resistant cell line, MKN45 cells were continuously exposed to increasing dosages (0.1-2 μM) of 5-FU over a year. The surviving cells were cloned and the resulting 5-FU resistant cell line was designated MKN45/F2R. MKN45/F2R routinely survive exposure to 2 μM 5-FU, and therefore, was maintained in culture medium containing this concentration of 5-FU. To eliminate the effects of 5-FU in our experiments, the resistant cells were cultured in a drug-free medium for at least two weeks before any procedure.

3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay. Cell growth was assessed by a standard 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay (Cell-Titer 96 aqueous nonradioactive MTT cell proliferation assay, Promega, Madison, WI), which detects viable dehydrogenase activity. Briefly, gastric cancer cells were seeded onto 96-well culture plates ($5x10^3$ cells/well). After 24 h, the cells were treated with various concentrations of drugs. After 72 h, 10 μl of a 5 mg/ml solution of MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, the plates were then incubated for 3 h at 37˚C. The growth medium was then replaced with 150 μl of dimethyl sulfoxide (Wako, Tokyo, Japan) per well, and the absorbance at 540 nm was measured using a Titertek Multiscan spectrophotometer.

Semiquantitive real-time polymerase chain reaction (RT-PCR). Total RNA was isolated using the Rneasy mini kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer's instructions. The quality and quantity of the total obtained RNA was determined by absorbance at 260 and 280 nm and was adjusted at a concentration of 0.10 μg/μl. The total prepared RNA served as the template in the first-strand cDNA synthesis using Ready-To-Go™ You-Prime-First-strand beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with 0.5 μg of Oligo(dT) 15 primer (Novagen, Darmstadt, Germany) according to the manufacturer's instructions. Each cDNA product was diluted 10-fold with RNase-free water to avoid inhibition of the RNA extraction or reverse transcription. For relative quantification by PCR, each cDNA product was analyzed at a final Mg^{2+} concentration of 3 mM in a LightCycler with version 3.5 software (Roche Molecular, Mannheim, Germany) by using a FastStart

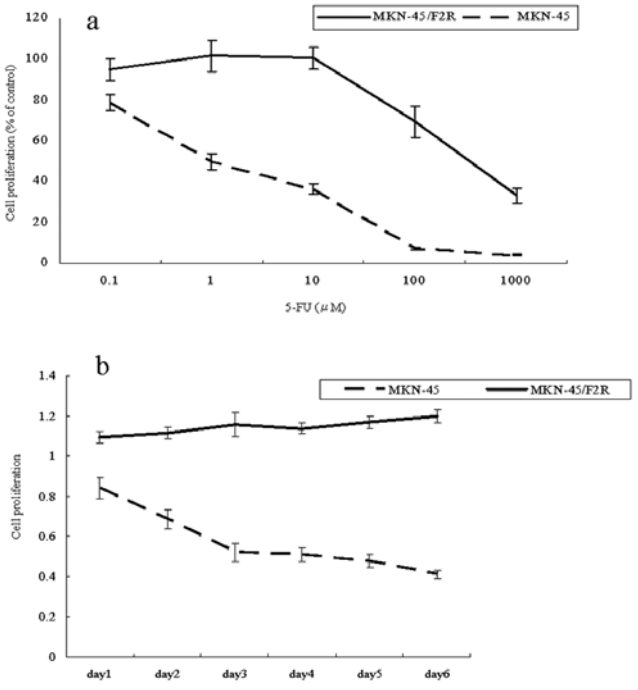

Figure 1. (a) *In vitro* sensitivity of the MKN45 and MKN45/F2R cells to 5-FU. Cells were cultured with various concentrations of 5-FU for 72 h. The relative number of living cells was measured by MTT assay. Each data point represents the mean ± standard deviation of at least three replications. The MKN45/F2R cells were more resistant to 5-FU than the MKN45 cells. (b) Growth curves of the MKN45/F2R and MKN45 parent cell line with 2 μM of 5-FU.

DNA Master SYBR Green I kit (Roche Diagnostics). For each primer pair, a standard curve was developed. The PCR conditions for TS, DPD, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 95˚C for 10 min for the initial denaturation, followed by 35 cycles of 10 sec at 95˚C, 10 sec at 68˚C, and 10 sec at 72˚C, followed by a melting program (at 58-95˚C) to check the proper melting temperature of the product. The PCR condition for OPRT was 95˚C for 10 min for the initial denaturation, followed by 40 cycles of 10 sec at 95˚C, 15 sec at 60˚C, and 9 sec at 72˚C. The relative gene expression was determined by the ratio of copy numbers for TS, DPD, OPRT, and GAPDH (initial control). Primers for TS, DPD, and GAPDH were from Search-LC GmbH (Heidelberg, Germany) and the primer for OPRT was from Roche Diagnostic (Tokyo, Japan).

Western blot analysis. Gastric cancer cells were harvested and lysed in a modified protein lysis buffer (10 mM Tris-HCl, pH 8.0, 135 mM NaCl, 1 mM EDTA, 10 mM CHAPS, 10 μg/ml aprotinin, 0.02 mM APMSF), and the protein concentration of the lysates was measured. Total cell protein extracts (15 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Readygels J (Bio-Rad, Hercules, CA, USA), and were electrophoretically transferred onto polyvinyl difluoride membranes. The membranes were blocked with 5% nonfat dried milk in phosphate buffered saline (PBS) containing 0.05% Tween-20. The filters were then incubated with primary antibodies against ß-actin (Sigma-Aldrich), TS, DPD, OPRT (provided by Taiho Pharma, Tokyo, Japan), and class III ß-tubulin (Chemicon

Table I. Comparison of drug resistance of the MKN45 and MKN45/F2R cells.

Drugs	MKN-45	MKN-45/F2R	fold
5-FU	2.46±1.29	386.2±275.0	157
Cisplatin	2.41±0.46	4.0±0.32	1.66
Oxaliplatin	0.59±0.46	3.21±1.24	5.44
SN38	5.62±1.35	15.8±4.91	2.81
Paclitaxel	0.16±0.11	>10	>61.1
Docetaxel	0.027±0.019	>10	>369

The IC_{50} values and drug concentrations (μM) at which cell growth were inhibited by 50% of each chemotherapeutic drug 72 h after treatments is shown.

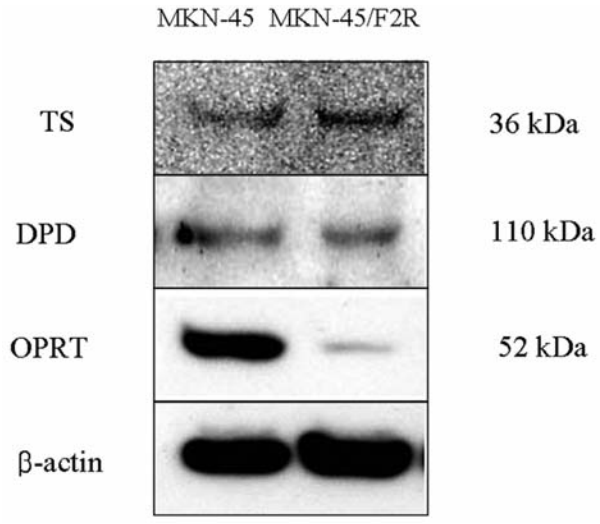

Figure 2. Western blot analysis of the expressions of thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyltransferase (OPRT), and ß-actin (ACTB) in the MKN45 and MKN45/F2R cells. OPRT expression decreased in the 5-FU resistant cells compared to the parent cells.

International, Temecula, CA, USA). Other primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies were diluted as recommended by the particular manufacturer. Membranes were then washed with PBS-T buffer (PBS with 0.1% Tween-20) and incubated with the appropriate secondary antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International, Buckhinghamshire, UK).

Measurement of TS, DPD, and OPRT activities. Tumors were thawed to 4˚C and placed in 3 volumes of 0.2 M Tris-HCl buffer, pH 7.4, containing 20 mM 2-mercaptoethanol, 15 mM cytidine 5'-monophosphate and 100 mM sodium fluoride. Tissues were homogenized by sonication. TS activity was quantified using a tritiated 5-fluorodeoxyuridine diphosphate binding assay (18). For the DPD measurement, the activity was determined by a previously described catalytic assay (19), and OPRT activity was determined using a previously described 5-FU phosphorylation assay (20).

Transfection and small interfering RNA experiments. MKN45 cells were cultured in a medium without antibiotics 24 h before transfection to produce 50-70% confluence. Cells were transfected with small interfering RNA (siRNA) oligonucleotide using Lipofectamine™ 2000 (Invitrogen) to result in a final RNA concentration of 40 nmol/l in serum-free Opti-MEM (Invitrogen) according to the manufacturer's instructions. At 6 h after transfection, the medium was replaced with RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. After an additional 48 h, total RNA or protein was extracted, and the MTT assay was performed. The siRNA oligonucleotides for OPRT (predesigned siRNA, ID number 8435) were purchased from Ambion (Austin, TX, USA).

Results

Establishment of a resistant cell line and its characterization. To obtain 5-FU resistant cells, the MKN45 cells were treated with increasing concentrations of 5-FU from 0.1-2 μM for over a year and a resistant clone, designated as MKN45/F2R was established from a culture. Initially, the growth and the IC_{50} of chemotherapeutic agents were examined including 5-FU, cisplatin, oxaliplatin, SN38, paclitaxel, and docetaxel. As shown in Fig. 1a, the IC_{50} of the MKN45 and MKN45/F2R cells was 2.46 and 386.2 μM, respectively, representing a 157-fold increased resistant capacity of the clone compared to the parent cell line. The growth curves of the MKN45/F2R and MKN45 parent cell line with 2 μM of 5-FU are displayed in Fig. 1b. Growth of the cell line in the absence of 5-FU was not appreciably different (data not shown).

To examine the cross-resistant capacity with other chemotherapeutic agents, the IC_{50} with each agent was examined. Interestingly, although MKN45/F2R cells acquired slight tolerance to cisplatin (1.66-fold), SN38 (2.81-fold), and oxaliplatin (5.44-fold), the cells acquired pronounced resistant capacity towards paclitaxel, deocetaxel, and 5-FU (Table I).

Changes in the expression levels of the TS, DPD, and OPRT metabolic enzymes. Since TS, DPD, and OPRT are known to play key roles in the function of 5-FU, the expression of mRNA, proteins, and their enzyme activities were examined to understand the mechanism of the acquired resistance of MKN45/F2R cells. The expression levels of the TS, DPD, and OPRT proteins were investigated by Western blot analysis. As shown in Fig. 2, the expression of TS protein was clearly detected at 36 kDa, and was enhanced 1.1-fold in the MKN45/F2R cell line compared to the MKN45 parent cell line. DPD was detected at 110 kDa and there was no significant difference in the expression between the two cell lines. However, the expression of OPRT at 52 kDa was markedly decreased to 7% in the MKN45/F2R cell line compared to the MKN45 parent cell line.

To examine whether the expression changes in the 5-FU metabolic pathway genes were controlled at the transcriptional level, the mRNA levels were analyzed by RT-PCR. As shown in Fig. 3, the changes in mRNA expression were similar to those of protein expression detected by Western blotting. The

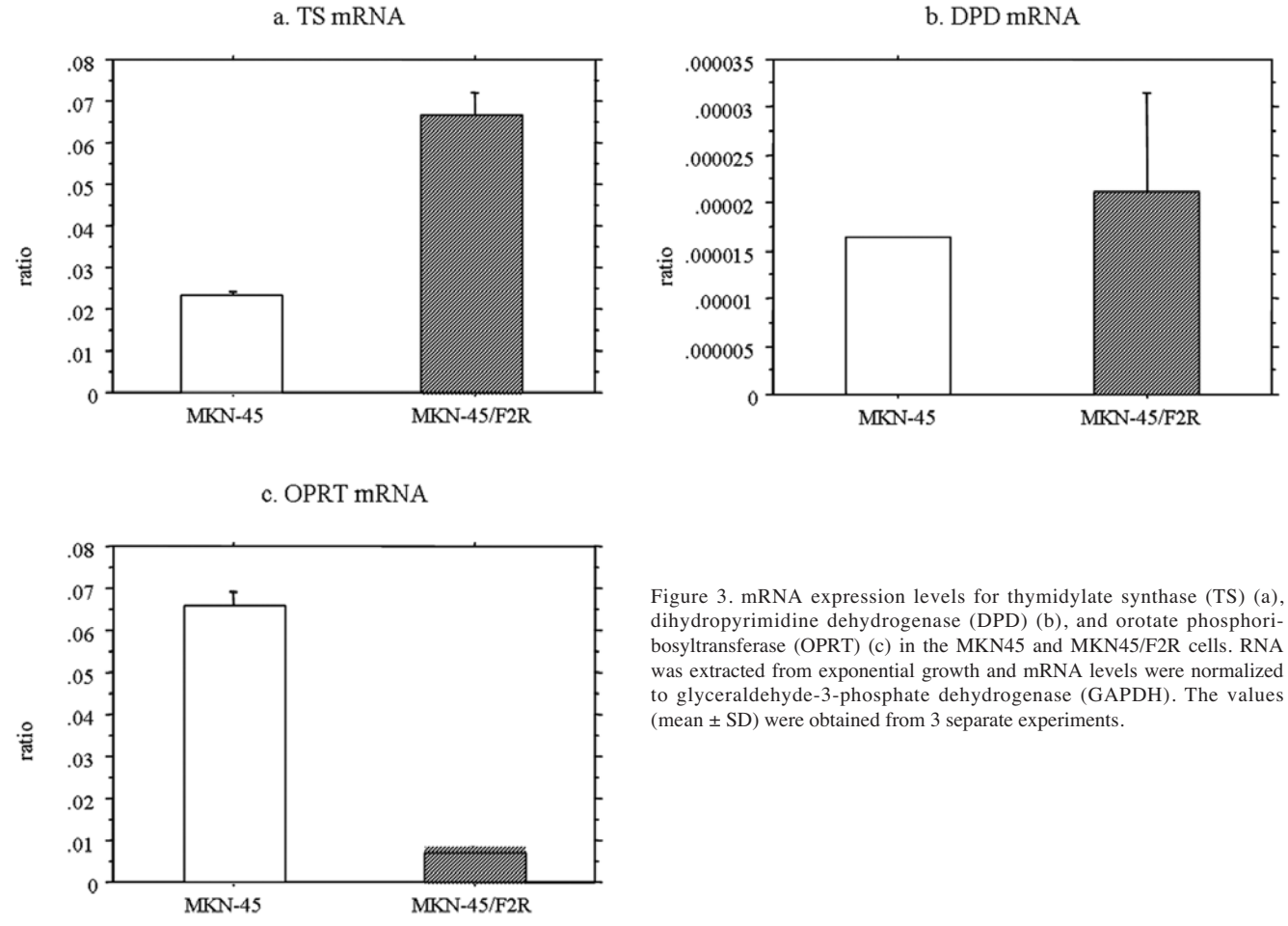

Figure 3. mRNA expression levels for thymidylate synthase (TS) (a), dihydropyrimidine dehydrogenase (DPD) (b), and orotate phosphoribosyltransferase (OPRT) (c) in the MKN45 and MKN45/F2R cells. RNA was extracted from exponential growth and mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The values (mean ± SD) were obtained from 3 separate experiments.

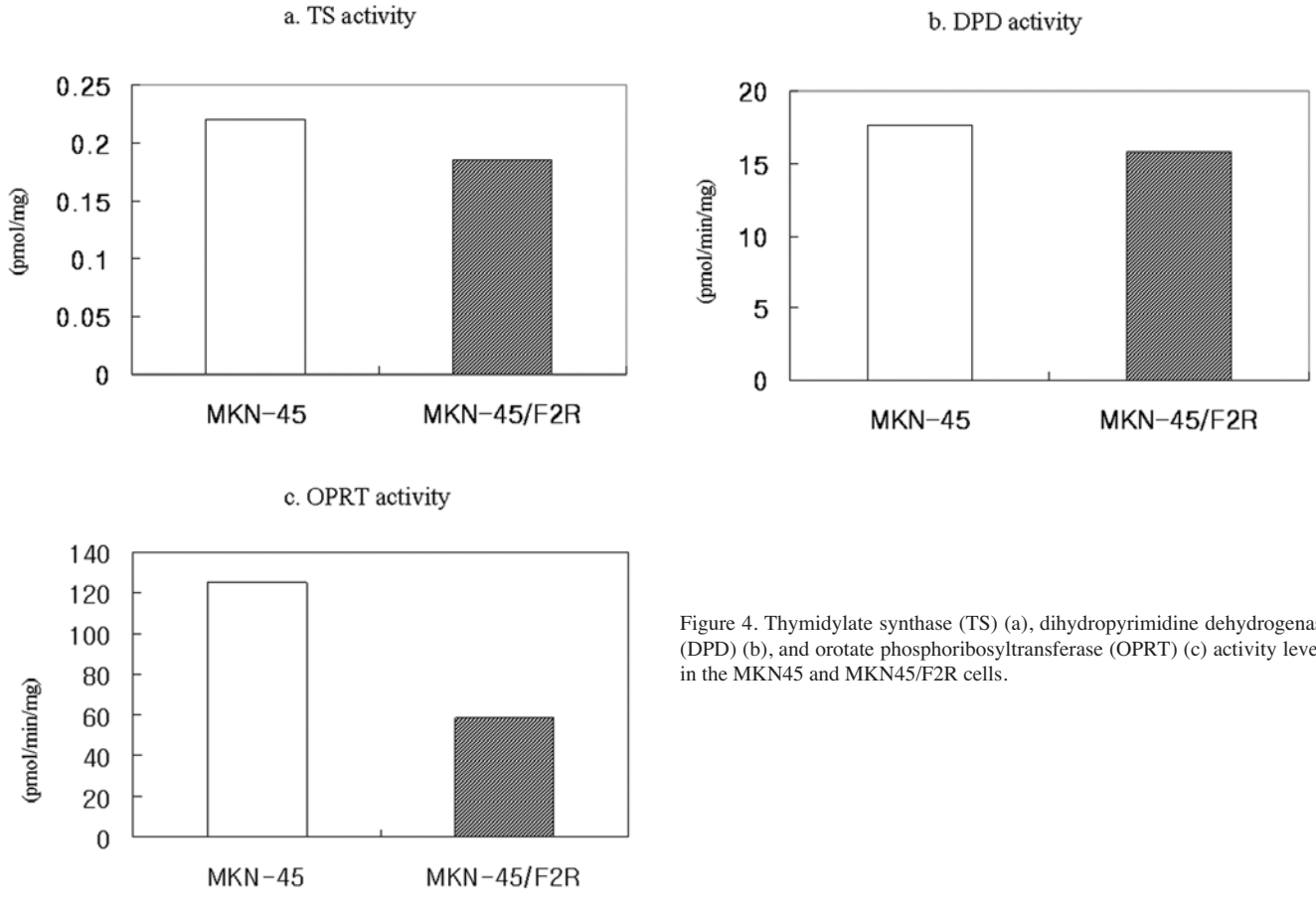

Figure 4. Thymidylate synthase (TS) (a), dihydropyrimidine dehydrogenase (DPD) (b), and orotate phosphoribosyltransferase (OPRT) (c) activity levels in the MKN45 and MKN45/F2R cells.

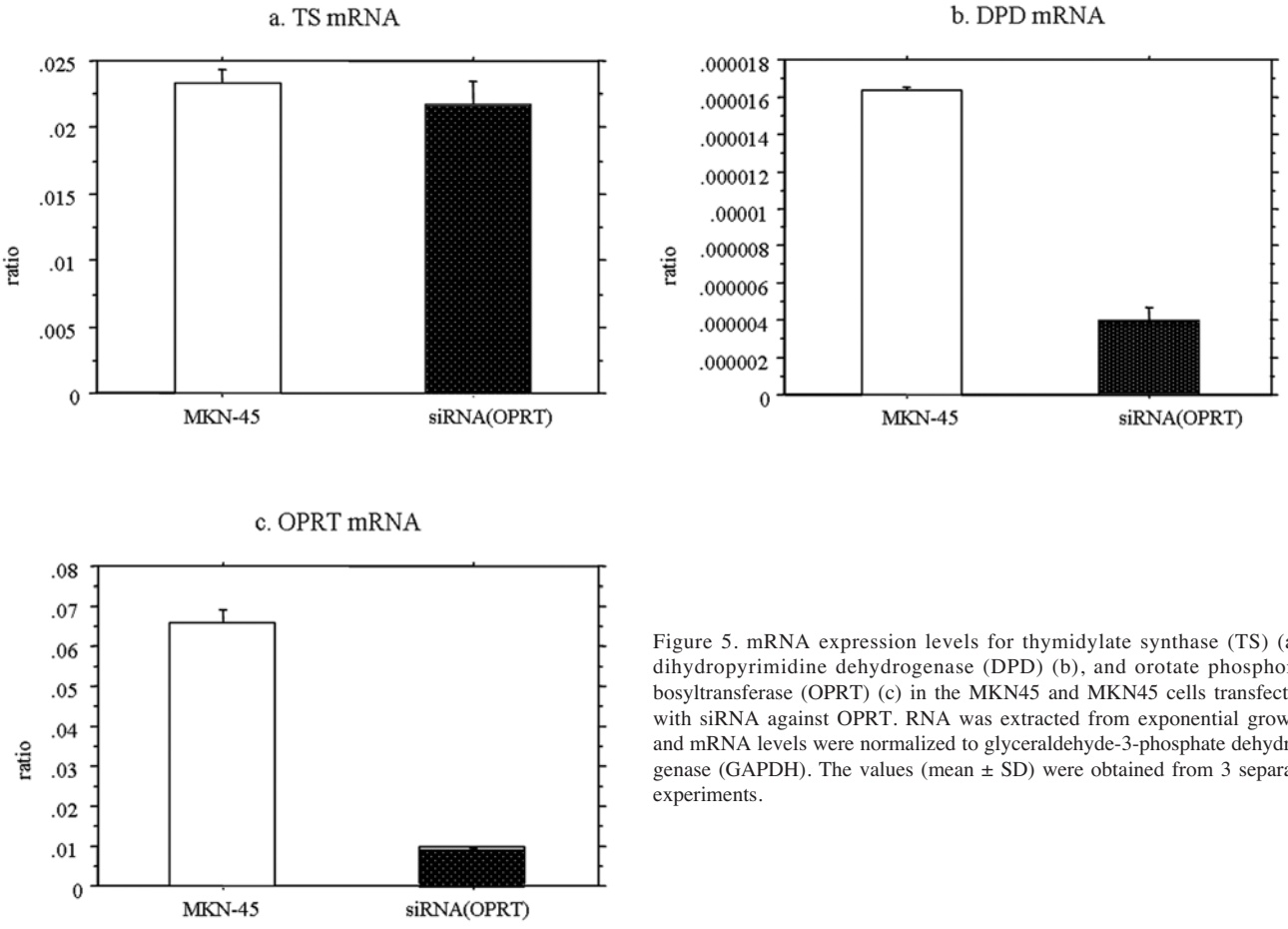

Figure 5. mRNA expression levels for thymidylate synthase (TS) (a), dihydropyrimidine dehydrogenase (DPD) (b), and orotate phosphoribosyltransferase (OPRT) (c) in the MKN45 and MKN45 cells transfected with siRNA against OPRT. RNA was extracted from exponential growth and mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The values (mean ± SD) were obtained from 3 separate experiments.

Table II. Comparison of drug resistance of the MKN45 and MKN45 cells transfected with siRNA against OPRT.

Drugs	MKN-45	siRNA(OPRT)	fold
5-FU	2.46±1.29	38.8±6.53	15.80
Cisplatin	2.41±0.46	1.47±0.36	0.61
Oxaliplatin	0.59±0.46	0.53±0.22	0.90
SN38	5.62±1.35	7.1±3.0	1.23
Paclitaxel	0.16±0.11	0.0053±0.002	0.03
Docetaxel	0.027±0.019	0.020±0.017	0.74

The IC_{50} values (μM) of each chemotherapeutic drug at 72 h after treatments are shown.

expression level of TS mRNA was enhanced 2.87-fold and that of DPD was 1.23-fold in the MKN45/F2R cell line compared to the MKN45 parent cell line. More importantly, the mRNA expression of OPRT was markedly decreased to 12.4% in the MKN45/F2R cell line compared to the MKN45 parent cell line, confirming that the expression is regulated at the posttranscriptional level.

Furthermore, the activities of these enzymes were also measured in these cell lines. As shown in Fig. 4, the activity of OPRT was 58.83 and 125.36 pmol/min/mg in the MKN45/F2R and MKN45 cell lines, respectively, indicating that the activity was decreased 46.9% in the 5-FU resistant cells. The activities of TS and DPD were not altered in the 5-FU resistant cells compared to the parent cells.

These results strongly indicated that the decreased expressions of mRNA, protein, and the activity of OPRT can be one of the main causes for the chemoresistant character of MKN45/F2R cell line.

Transfection and siRNA experiments. To confirm that the decreased expression of OPRT gene directly induces 5-FU resistance capacity, the siRNA directed against OPRT was transfected to MKN45 parent cell line and the sensitivity was analyzed. Before analyzing the IC_{50}, mRNA expressions of OPRT, TS, and DPD were analyzed to confirm that the OPRT gene was down-regulated. Cells were harvested at 48 h after transfection, and extracts were prepared and analyzed by semiquantitative RT-PCR.

Although the expression level of TS was not altered after transfection with siRNA in the MKN45 cell line compared to the MKN45 parent cell line without transfection, DPD and OPRT gene expressions were markedly decreased to 16.9 and 14.4%, respectively, confirming that the OPRT gene expression was down-regulated by siRNA (Fig. 5).

Next, the IC_{50} of 5-FU and other chemotherapeutic agents were examined after treatment with each drug for 72 h to confirm that the decreased OPRT expression by siRNA interferes with sensitivity towards other drugs. As expected, the IC_{50} of 5-FU in MKN45 transfected with siRNA increased to 38.8 μM, *i.e.* 15.8-fold resistance was obtained after transfection, indicating that the decreased OPRT expression correlated with the decreased response to 5-FU (Table II).

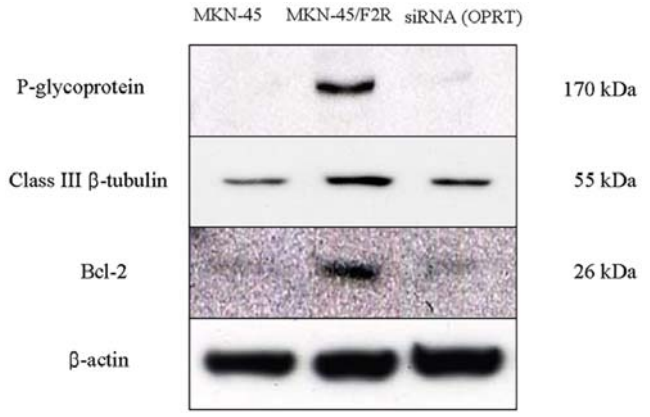

Figure 6. Expression of P-glycoprotein, class III ß-tubulin, and Bcl-2 proteins in the MKN45 and MKN45/F2R cells. Protein expression was not significantly changed in the MKN45 cells transfected with siRNA compared to the MKN45 parent cell line. Conversely, the expressions of these proteins were increased to 95.9-, 2.2-, and 2.1-fold, respectively compared to the MKN45 parent cell line.

The sensitivities of cisplatin, oxaliplatin, SN38, and docetaxel was not altered significantly; however, paclitaxel sensitivity was markedly increased in the MKN45 cells transfected with siRNA.

Mechanism of crossresistance to taxanes in MKN45/F2R cells. To elucidate the mechanism of crossresistance to taxanes in the MKN45/F2R cells, we examined the expressions of several genes by Western blot analysis. Expressions of P-glycoprotein (a product of the MDR1 gene), class III ß-tubulin and Bcl-2, which are known to be involved in taxane resistance, were examined in the MKN45 parent cell line, MKN45/F2R cells, and MKN45 cells transfected with siRNA for OPRT. The expressions of these genes were not significantly changed in the MKN45 cells transfected with siRNA compared to the MKN45 parent cell line. Conversely, the expressions of proteins for P-glycoprotein, class III ß-tubulin, and Bcl-2 increased 95.9-, 2.1-, and 2.2-fold, respectively, in the MKN45/F2R cells compared to the MKN45 parent cells (Fig. 6).

These results indicated that the alterations of expressions of these genes were not directly related to down-regulation of OPRT gene expression; however, they might be related to other mechanism including epigenetic changes of these genes.

Discussion

5-FU metabolism has been extensively studied, as have the enzymes involved including TS, DPD, and OPRT. Inhibition of RNA and DNA function by 5-FU is thought to be controlled by three pathways: the primary pathway is phosphorylation of 5-FU by OPRT into FUMP, second is the conversion of 5-FU by 5-fluorouridine phosphorylase into 5-fluorouridine, and third is phosphorylation of 5-fluorouridine by 5-fluorouridine kinase into FUMP. 5-Fluorouridine disphosphate is then converted into 5-fluorouridine triphosphate and subsequently taken up by RNA. Although the precise mechanisms of these pathways are not well understood, OPRT has received attention as another important factor predicting the effects of 5-FU (16). OPRT directly metabolizes 5-FU to FUMP in the presence of 5-phosphoribosyl-1-pyrophosphate and thus, is believed to be correlated with a higher sensitivity to 5-FU (16). Furthermore, we have previously demonstrated that the sensitivity to 5-FU is increased in OPRT transfected cells *in vitro* and *in vivo* (21).

In the present study, we established a 5-FU resistant cell line derived from gastric cancer cells for the purpose of studying mechanisms of drug resistance in gastric cancer. MKN45/F2R cell line showed 157-fold resistance to 5-FU, and we detected a marked decrease in OPRT protein expression in this cell line, although there were no remarkable changes in the expressions of TS and DPD proteins between the MKN45 and MKN45/F2R cell lines. The activities of TS, DPD, and OPRT closely correlated with the protein expression in both cell lines. Decreased expression of 5-FU anabolizing enzymes including OPRT gene has been previously described in 5-FU-resistant gastric cancer cells (21,22). To clarify the interaction between OPRT expression and 5-FU sensitivity, we used the siRNA technique. The MKN45 cells were transfected with siRNAs directed against OPRT; OPRT-knockout MKN45 cells showed resistance to 5-FU (15.8-fold) compared to the control cells. Decreased OPRT gene expression did not induce resistance in MKN45 cells to other drugs including paclitaxel and docetaxel. However, the sensitivity to paclitaxel was increased in the MKN45 cells transfected with siRNA. It is still unclear whether the transfection process is related or not.

These results strongly indicate that OPRT decrease plays an important role in the resistance of gastric cancer cells to 5-FU. Interestingly, DPD expression was also decreased in OPRT-knockout MKN45 cells, and the phenomenon might be the result of transfection with siRNA against OPRT.

Tumor cells that are resistant to a certain anticancer drug often acquire resistance to other drugs, and overexpression of P-glycoprotein, which plays a role in the drug transport, can cause increased efflux of doxorubicin and paclitaxel (23). In the present study, MKN45/F2R showed strong crossresistance to paclitaxel and docetaxel, and expression of P-glycoprotein was increased in the resistant cells, implicating the expression of P-glycoprotein as one of the determinants in the taxane resistance of MKN45/F2R cells. Since class III ß-tubulin is associated with a resistance to taxanes (24,25), class III ß-tubulin expression in the 5-FU resistant cells was also examined. Higher expression of class III ß-tubulin was detected in the MKN45/F2R cells than in the MKN45 parent cells. This result demonstrates that an increased expression of class III ß-tubulin also contributes to paclitaxel and docetaxel resistance. Bcl-2 may be another factor that contributes to this crossresistance, since enhanced expression of Bcl-2 blocks apoptosis and is associated with resistance to several anticancer drugs including paclitaxel and docetaxel (26,27).

Based on the above considerations, we suggest that increased expression of Bcl-2 in the 5-FU resistant cell line inhibits paclitaxel- and docetaxel-induced apoptosis, conferring taxane resistance to MKN45/F2R. Our results demonstrate that crossresistance to taxanes in the 5-FU resistant cell line results from increased expressions of

P-glycoprotein, class III ß-tubulin, and Bcl-2, probably by acting synergistically.

Further studies including the experiments of cDNA microarray and proteomic analysis will be helpful to clarify other factors of resistance to 5-FU and taxanes. Nevertheless, the present results strongly indicate that OPRT decrease plays an important role in the resistance of gastric cancers to 5-FU chemotherapy and increases the expression of several genes correlated with the resistance to taxanes, suggesting the possibility of personalized chemotherapy including the prediction of response and adverse effects.

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