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Orotate phosphoribosyltransferase expression level in tumors is a potential determinant of the efficacy of 5-fluorouracil

Etsuko Sakamoto [a,*], Hideki Nagase [a], Takashi Kobunai [a], Shinji Oie [a], Toshinori Oka [a], Masakazu Fukushima [a], Tatsuzo Oka [b]

[a] *Personalized Medicine Research Laboratory, Taiho Pharmaceutical Co., Ltd., 224-2 Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-0194, Japan*
[b] *Department of Veterinary Medicine, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan*

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Abstract

Although the intratumoral expression levels of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) are known to affect the antitumor activity of 5-fluorouracil (5-FU), the importance of orotate phosphoribosyltransferase (OPRT) has remained unclear. This study investigated the relationship between intratumoral OPRT expression and the antitumor activity of 5-FU using human NCI60 cell lines with similar levels of TS and DPD messenger RNAs, as well as 31 tumor xenografts. The OPRT mRNA level was positively correlated with the 5-FU efficacy in these cell lines. *In vitro*, the 50% growth-inhibitory concentrations of 5-FU were closely correlated with the OPRT mRNA levels in cancer cell lines with similar levels of TS mRNAs when combined with a DPD inhibitor. Moreover, downregulation of OPRT with small-interfering RNA decreased the sensitivities of the cultured tumor cells to 5-FU. These results suggest that the OPRT expression level in tumors is an additional determinant of the efficacy of 5-FU.

Keywords: 5-FU; DPD; OPRT; TS

The antimetabolite 5-fluorouracil (5-FU) exerts its anticancer effect mainly by inhibiting thymidylate synthase (TS) via the formation of a stable ternary complex of TS, 5,10-methylenetetrahydrofolate (MTHF) and 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), which is the active form of 5-FU [1]. Therefore, a low level of TS, and high levels of MTHF and FdUMP, should lead to strong inhibition by 5-FU.

TS is a key enzyme in the synthesis of deoxythymidine nucleotides, and its inhibition is thought to deplete deoxythymidine triphosphate, resulting in the suppression of DNA synthesis [1]. Johnston et al. reported that both the messenger RNA (mRNA) and protein levels of TS were associated with the responses of colorectal and gastric tumors to 5-FU [2]. More than 80% of any dose of 5-FU is rapidly catabolized, primarily in the liver, where dihydropyrimidine dehydrogenase (DPD), which is the rate-limiting enzyme in 5-FU catabolism, is abundant [1]. The DPD level is thus an important indicator of the efficacy of 5-FU. Ichikawa et al. reported that the response rate to a fluoropyrimidine-based protocol was 75% in colorectal tumors with low levels of DPD and TS mRNA, and the median survival time was longer in patients with these tumors than in patients with tumors with high DPD and TS [3]. These reports suggest that the expression levels of TS and DPD are determinants of the efficacy of 5-FU; however, a method of predicting its efficacy based on these levels has not yet been established, possibly because these two factors are not the only determinants.

5-FU must be phosphorylated to exert an antitumor effect. There are three pathways of 5-FU phosphorylation, and its direct conversion to 5-fluorouridine 5′-monophosphate by orotate phosphoribosyltransferase (OPRT) is predominant in tumor tissues [4]. OPRT is one component of

* Corresponding author. Fax: +81 88 665 6554.
E-mail address: etsu-toda@taiho.co.jp (E. Sakamoto).

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UMP synthase; the other component is orotidine-5′-phosphate decarboxylase (ODC), which is involved in *de novo* pyrimidine biosynthesis [5]. OPRT and ODC are encoded by a single mRNA [6], and the levels of OPRT transcripts and activity are elevated in various tumor tissues [7–9].

There is some evidence that the OPRT level affects 5-FU sensitivity [10–12]. Colorectal cancer patients with high tumor OPRT activity were reported to have better disease-free and overall survival when treated with 5-FU-based adjuvant chemotherapy [10]. Moreover, the colorectal tumors that responded to tegafur-uracil plus leucovorin (UFT/LV) therapy had a higher OPRT/DPD ratio than non-responding tumors [11]. By contrast, Ishida et al. found that the level of OPRT transcripts was not related to the efficacy of 5-FU in colorectal cancers [13]. Thus, there are conflicting data on the relationship between OPRT expression and the efficacy of 5-FU.

5-FU has been used for more than 40 years to treat various cancers, although the response rates when it is used as a single agent are less than 20% [1]. To increase anticancer activity, new 5-FU-based drugs, such as S-1 and capecitabine, have been developed. S-1, in particular, has been shown to elicit a good response in gastric cancer, with a 46.5% response rate [14]. Yet there are still patients who do not benefit from these drugs, and those who suffer from adverse effects. It is therefore of considerable importance to establish a way to identify likely responders to these drugs.

In the current study, we investigated the relationship between the level of OPRT mRNA and the efficacy of 5-FU in human cancer cell lines *in vitro* and *in vivo*. We used cancer cell lines with similar levels of expression of TS and DPD, in order to reduce their influence on 5-FU efficacy. Moreover, we examined the effect of downregulating OPRT with small-interfering RNA (siRNA) on the cytotoxicity of 5-FU.

Materials and methods

Gene expression and cytotoxicity for NCI60 cell lines. Data on the cytotoxic effect of 5-FU on 60 human cancer cell lines were downloaded from NCI database (http://dtp.nci.nih.gov). Gene expression data for the NCI60 cell lines were obtained with Affymetrix high-density Hu6800 arrays and downloaded from the Broad Institute (http://www.broad.mit.edu/tools/data.html) [15], and were processed with Microarray Suite 5.0 software (Affymetrix, Santa Clara, CA). The data were imported into GeneSpring software (Agilent Technologies, Inc., Santa Clara, CA) and normalized by the median of the chip.

Cell lines. We used 31 cell lines to evaluate the antitumor activities of drugs. These cell lines were derived from cancers of the breast (MC-5, H-31, MC-2, MX-1, MDA-MB-435SHM and MDA-MD-231), colon (KM12C, HCT-15, KM20C, COL-1, KM12C/FU and CO-3), lung (GT3TKB, LC-11, Lu-99, LX-1, LC-6, Lu-134 and Lu-130), pancreas (PAN-3, PAN-4, PAN-12, H-48, MIAPaCa-2 and BxPC-3) and gastric system (AZ-521, SC-2, ST-40, 4-1ST, SC-4 and OCUM-2MD3). In addition, five human cancer cell lines (lung PC-9, prostate DU145, bladder TSU-Pr1, pancreas MIAPaCa-2 and gastric OCUM-2MD3) were grown in RPMI1640 medium (Sigma–Aldrich Co., St. Louis, MO) with 10% fetal bovine serum (MP Biomedicals, Inc., Aurora, OH).

Drugs. 5-FU was purchased from Wako Pure Chemical Industries (Osaka, Japan). S-1 and 5-chloro-2,4-dihydroxypyridine (CDHP) were synthesized by Taiho Pharmaceutical Co. (Tokyo, Japan). 5′-Deoxy-5-fluorouridine (5′-DFUR) was purchased from Nippon Roche K. K. (Tokyo, Japan).

Cytotoxicity test. Cells were seeded in a 96-well plate at a density of $\sim 1–2 \times 10^3$ per well. After overnight incubation, they were treated with the drugs for 72 h. The number of viable cells was estimated using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) [16]. The 50% growth-inhibitory concentration (GI_{50}) values were calculated using XLfit software (ID Business Solutions, Guildford, UK). We confirmed that the control cells grew exponentially for 72 h under these experimental conditions (data not shown).

Antitumor activity of S-1 and 5′-DFUR against human tumor xenografts. The cancer cells were inoculated subcutaneously into male nude mice. S-1 and 5′-DFUR were administered orally at maximum tolerated doses of 10 and 150 mg/kg, respectively, once daily for 14 consecutive days [17]. The relative tumor volume (RTV) was calculated as follows: RTV = (tumor volume on day 15)/(tumor volume on day 0). The antitumor effect was calculated as follows: tumor growth inhibition rate (%) = (1 – mean RTV of treatment group/mean RTV of untreated group) × 100. The tumors of the control group were harvested on day 15 for mRNA analysis.

Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR). Quantitative real-time RT-PCR was performed on a PRISM 7900 sequence detector (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). The gene expression levels in tumor xenografts were normalized by the geometric mean of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta actin (ACTB) levels, and those in the cultured cells were normalized by GAPDH. The primers and TaqMan probes for OPRT, TS, DPD, GAPDH and ACTB were prepared with Assay-on-Demand gene expression products (Applied Biosystems).

Silencing of OPRT. The siRNA oligonucleotides for OPRT (NM_000373) and siCONTROL RISC-free siRNA as a control were obtained from Dharmacon Research (Chicago, IL). The cells were plated into flasks at a density of 1.25×10^4 per cm^2. After overnight incubation, they were treated with siRNA complexed to Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Western blotting. Total cellular protein was separated by SDS–PAGE on NuPAGE 10% Bis–Tris Gels (Invitrogen) in MOPS running buffer (Invitrogen), and transferred onto a nitrocellulose membrane. OPRT was probed with an anti-OPRT polyclonal antibody [18], and visualized using HRP-conjugated antibodies (DakoCytomation Co., Kyoto, Japan) and an ECL advance Western blotting detection kit (Amersham Biosciences, Buckinghamshire, UK). Chemiluminescent signals were detected with an image analyzer (LAS-3000 Mini; Fuji Film Co. Ltd., Tokyo, Japan).

Statistical analysis. To evaluate the correlation between gene expression levels and drug susceptibilities, gene expression data were normalized by the median expression level and compared with log-transformed GI_{50} data. Correlations were determined from the Pearson's correlation coefficient. JMP (SAS Institute Inc., Cary, NC) was used for statistical analysis.

Results

Relationship between OPRT mRNA level and 5-FU cytotoxicity in NCI60 cell lines

To examine the relationship between the level of OPRT mRNA and the cytotoxicity of 5-FU, we downloaded gene expression and 5-FU cytotoxicity data for 60 human cancer cell lines in an NCI60 panel. As shown in Fig. 1A, the level of OPRT mRNA was correlated with the GI_{50} value for 5-FU ($P = 0.05$), although the correlation coefficient was −0.255. To reduce the influence of TS and DPD on 5-FU cytotoxicity, we selected cell lines that contained

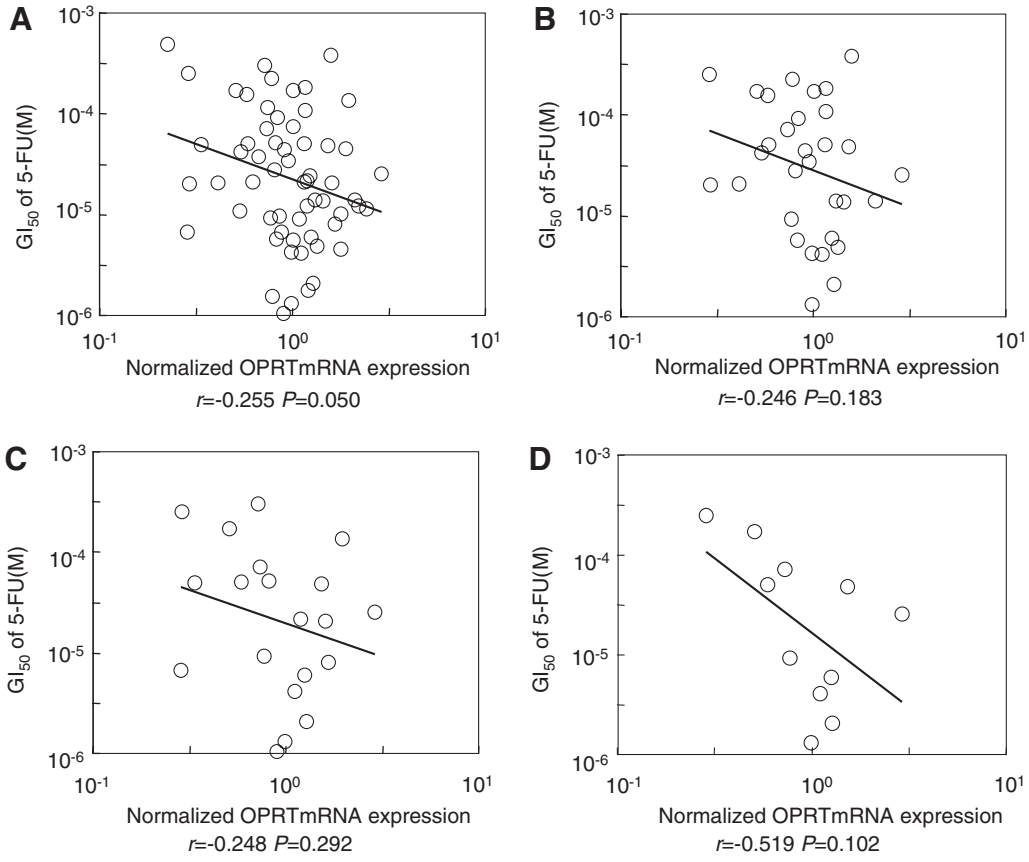

Fig. 1. Relationship between the level of OPRT mRNA and the cytotoxic effects of 5-FU in (A) 60 human cancer cell lines of an NCI panel, (B) 31 cell lines selected for their similar TS mRNA levels, (C) 20 cell lines selected for their similar DPD mRNA levels and (D) 11 cell lines selected for their similar TS and DPD mRNA levels. The data for OPRT mRNA levels measured with an oligonucleotide microarray were normalized by the median of the chip, and then divided by the median expression level.

TS or DPD mRNAs within the upper and lower limits of 1.5 times the median value in the 60 cell lines. In total, 31 cell lines were selected on the basis of the expression of TS, and there was no correlation between the level of OPRT mRNA and the GI_{50} value for 5-FU in these lines (Fig. 1B). Similarly, there was no correlation between the level of OPRT mRNA and the GI_{50} value in 20 cell lines selected on the basis of the expression of DPD (Fig. 1C). However, when we selected 11 cell lines that contained both TS and DPD mRNAs within the upper and lower limits of 1.5 times the median expression values in the 60 cell lines, the correlation coefficient between the level of OPRT mRNA and the GI_{50} value for 5-FU was higher than in all 60 cell lines (-0.519), although the difference did not reach statistical significance ($P = 0.102$; Fig. 1D).

Relationship between OPRT mRNA level, 5′-DFUR and S-1 in human tumor xenografts

We found no relationship between the levels of OPRT mRNA and the antitumor activity of 5-FU-based drugs, 5′-DFUR and S-1, in 31 human tumor xenografts (Fig. 2A and C). The levels of TS and DPD transcripts varied 37-fold and 1.4×10^5-fold, respectively, in the 31 human tumors (data not shown). Therefore, we focused on seven of the 31 cell lines, namely, those in which the TS mRNA was within twice the upper and lower limits, and the DPD mRNA was within seven times the upper and lower limits of each median expression level, in order to reduce the variation of expression of TS and DPD. As shown in Fig. 2B, the level of OPRT mRNA was positively correlated with tumor growth inhibition by 5′-DFUR in the seven cell lines ($r = 0.759$, $P = 0.048$). In addition, the expression of OPRT also tended to be positively correlated with tumor growth inhibition by S-1 in the seven cell lines ($P = 0.081$), and the correlation coefficient was higher in these lines ($r = 0.699$) than in the 31 cell lines as a whole (Fig. 2D).

Relationship between OPRT mRNA level and 5-FU cytotoxicity when DPD activity was inhibited in cell lines with similar TS mRNA levels

We next investigated the relationship between the level of OPRT mRNA and the cytotoxic effect of 5-FU *in vitro*. To exclude the influence of TS expression on the efficacy of 5-FU, we selected five human cancer cell lines (MIAPaCa-2, OCUM2-MD3, TSU-Pr1, PC-9 and

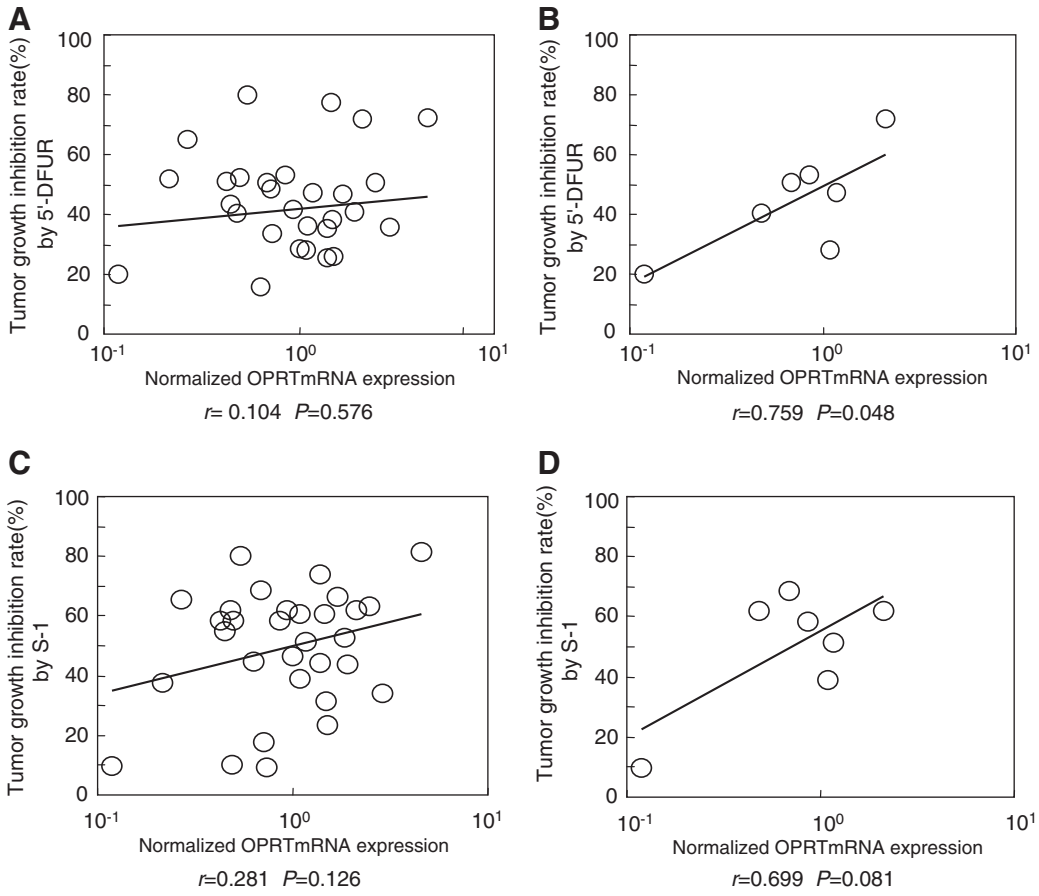

Fig. 2. Relationship between expression of OPRT mRNA and antitumor activity of (A, B) 5′-DFUR and (C, D) S-1 in (A, C) 31 human tumor xenografts and (B, D) seven tumor xenografts selected for their similar TS and DPD mRNA levels. OPRT mRNA levels were measured by quantitative real-time RT-PCR and normalized using the geometric mean of the levels for GAPDH and ACTB, and then divided by their median expression level.

DU145) with similar levels of TS mRNA. In these cell lines, the TS mRNA levels normalized by the levels of GAPDH ranged from 0.11 to 0.32 (Fig. 3A), the DPD levels ranged from 0.05 to 1.29, and the OPRT levels from 0.25 to 1.98. We used the DPD inhibitor CDHP to reduce the variation in DPD expression, and its influence on the cytotoxicity of 5-FU. CDHP at 70 μM completely inhibited the DPD activity without cytotoxicity [19]. In MIAPaCa-2 cells, which have high levels of DPD mRNA, the GI_{50} concentrations of 5-FU alone and in combination with CDHP were 13 and 7.2 μM, respectively, and CDHP treatment enhanced the cytotoxicity of 5-FU 1.8-fold (Fig. 3B). CDHP treatment also enhanced the cytotoxicity of 5-FU up to 1.3-fold in the other four cell lines. The GI_{50} values for 5-FU administered with CDHP were closely related to the levels of OPRT transcripts in the five cell lines ($r = 0.990$, $P = 0.001$; Fig. 3C).

Effect of downregulation of OPRT expression on 5-FU cytotoxicity

We examined the effect of downregulation of OPRT expression by siRNA on the cytotoxicity of 5-FU when the effects of TS and DPD were reduced. For this purpose, we selected the cell lines MIAPaCa-2 and OCUM-2MD3, because they had similar levels of TS mRNA, and we used CDHP to inhibit DPD activity. The cells were incubated with siRNA for 48 h, and then exposed to 5-FU plus CDHP for 72 h. The siRNA reduced OPRT mRNA levels in the MIAPaCa-2 and OCUM2-MD3 cells to 11% and 7%, respectively, of the control levels 48 h after transfection (data not shown), and the levels of OPRT protein were also reduced (Fig. 4A). siRNA treatment for 120 h reduced OPRT mRNA and protein levels in the MIAPaCa-2 cells to 48% (data not shown) and 22% (Fig. 4A), respectively, of those in the control cells, whereas OPRT mRNA in the OCUM-2MD3 cells recovered completely (data not shown), and OPRT protein was only reduced to 82% of that in the control cells (Fig. 4A). We confirmed that OPRT knockdown had no effect on the mRNA and protein levels of TS and DPD 48 h after transfection (data not shown). When the two cell lines were treated with OPRT siRNA, their GI_{50} values for 5-FU increased 28- and 4-fold, respectively (Fig. 4B and C). These results indicate the OPRT expression is positively related to cellular sensitivity to 5-FU.

Discussion

5-FU must be phosphorylated by an enzyme, such as OPRT, in order to show antitumor activity. However, the

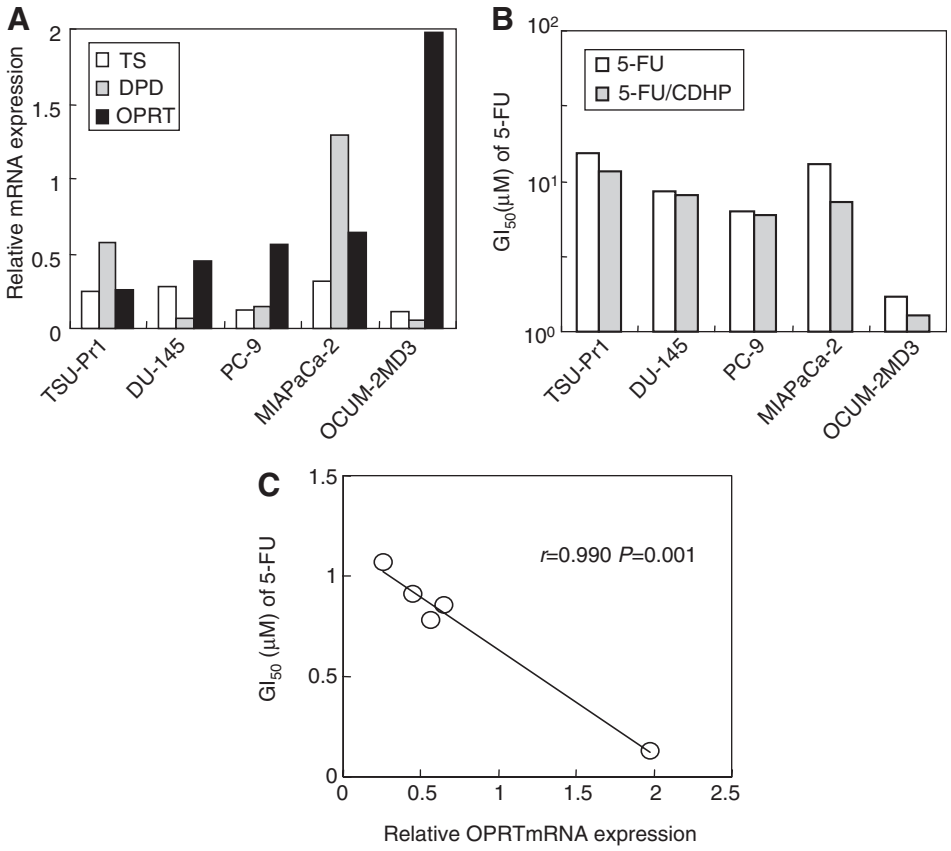

Fig. 3. (A) Levels of TS, DPD and OPRT mRNAs, (B) sensitivity to 5-FU and 5-FU/CDHP and (C) relationship between level of OPRT mRNA and cytotoxic effect of 5-FU/CDHP in MIAPaCa-2, OCUM2-MD3, TSU-Pr1, PC-9 and DU145 cells. The mRNA levels were measured by quantitative real-time RT-PCR and normalized by the level of GAPDH mRNA. The mRNA expression levels are the means of duplicate experiments. The cells were treated with various concentrations of 5-FU alone or 5-FU plus 70 μM CDHP for 72 h. GI_{50} values for 5-FU were calculated from three experiments.

importance of OPRT as a determining factor in the effectiveness of 5-FU has not been established. To clarify this relationship, we attempted to reduce the influence of TS and DPD. For this purpose, we selected human cancer cell lines with similar levels of TS and DPD mRNAs, and found that the OPRT mRNA level was positively correlated with the efficacy of 5-FU in these cell lines. Moreover, we confirmed this finding *in vitro*. Our results established that OPRT is a determinant of the efficacy of FU, in addition to TS and DPD.

We showed that the correlation between the OPRT mRNA level and the efficacy of 5-FU was stronger in cell lines with similar TS and DPD levels than in the cancer cell lines overall. The finding of Ishida et al. that the OPRT levels in colorectal tumors were not related to the efficacy of 5-FU-based drug [13] could be due to the influence of other factors. In fact, Ishida and colleagues found that the TS levels were correlated with efficacy. Our findings suggest that it is essential to determine the levels of TS, DPD, and OPRT expression in order to accurately predict the efficacy of 5-FU.

CDHP competitively inhibits DPD activity [20]. In agreement with Takechi et al. [21], we demonstrated that CDHP enhanced the sensitivity of cancer cell lines to 5-FU. This effect was strongest in the MIAPaCa-2 cell line, which had the highest level of DPD mRNA of the five cell lines. These results demonstrate that intracellular DPD activity attenuates the cytotoxicity of 5-FU, particularly when the cells have high levels of activity. S-1 contains tegafur (prodrug of 5-FU), CDHP, and potassium oxonate [22]. We previously reported that the DPD mRNA level was negatively correlated with the antitumor activities of 5-FU-based drugs administered in the absence of a DPD inhibitor, but not with the activity of S-1, which was administered with CDHP [17]. In the present study, the expression of OPRT was positively correlated with the antitumor activities of S-1, as well as with the cytotoxicity of 5-FU in combination with CDHP *in vitro*. Our results suggest that the level of OPRT is an important determinant of the anticancer activity of S-1, as the influence of DPD was reduced by CDHP.

Average tumor OPRT activities were previously reported to be 0.387 ± 0.168 nmol/min/mg protein and 0.120 ± 0.099 nmol/min/mg protein in 54 colorectal cancer patients [23] and in 20 gastric cancer patients [8], respectively. We previously reported that the tumor activities of TS and DPD were 0.071 ± 0.087 pmol/mg/protein and 140.8 ± 94.9 pmol/min/mg protein, respectively, in 458 gastric cancer patients [24]. These reports indicate that tumor OPRT levels are as variable as levels of TS and

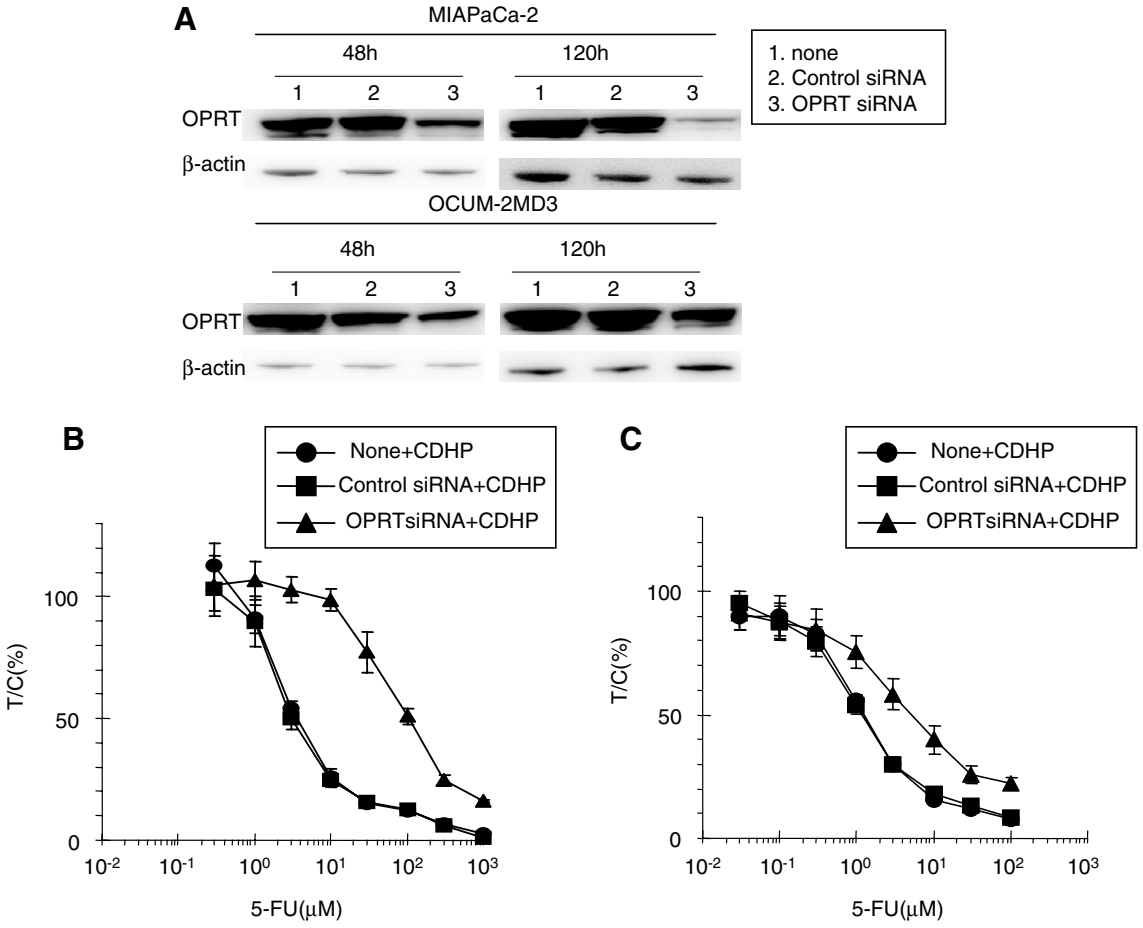

Fig. 4. (A) Effect of downregulation of OPRT with siRNA on the level of OPRT protein in MIAPaCa-2 and OCUM2-MD3 cells. (B, C) Sensitivities of (B) MIAPaCa-2 and (C) OCUM2-MD3 cells to 5-FU/CDHP. (A) OPRT protein was determined by Western blotting at 48 and 120 h after transfection. (B, C) Cells transfected with siRNA were treated with various concentrations of 5-FU along with 70 μM CDHP for 72 h. Each value is mean and standard deviation (bar) of three experiments.

DPD. Therefore, differences in the expression of these factors among patients might contribute to differences in sensitivity to 5-FU.

In this study, we demonstrated that OPRT expression in tumor was closely related with the anticancer activity, and that the downregulation of OPRT decreased the cellular sensitivity to 5-FU. In future clinical research, the significance of OPRT, in addition to TS and DPD, as a determinant of the efficacy of 5-FU-based chemotherapy, must be clarified by determining the expression levels of these factors in tumors.

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