**In vivo** evidence for a significant role of folylpolyglutamate synthase in combined chemotherapy with oral fluoropyrimidine, UFT or S-1, and leucovorin

SAYAKA TSUKIOKA, ETSUO SAKAMOTO, HIROAKI TSUJIMOTO, FUMIO NAKAGAWA, HITOSHI SAITO, JUNJI UCHIDA, MAMORU KINIWA and MASAKAZU FUKUSHIMA

Tokushima Research Center, Taiho Pharmaceutical Co., Ltd, 224-2 Hiraishi-ebisuno Kawauchi-cho, Tokushima 771-0194, Japan

Received November 24, 2010; Accepted January 14, 2011

DOI: 10.3892/or.2011.1206

**Abstract.** Combined chemotherapy with 5-fluorouracil and leucovorin (LV) has been widely used for the treatment of patients with colorectal cancer. Given that LV effects are attributable to increased levels of reduced folate in cancer cells, we attempted here to show the *in vivo* role of folylpolyglutamate synthetase (FPGS), which stabilizes intracellular reduced folate, in the anticancer activities of oral fluoropyrimidines, UFT or S-1, combined with LV. To this end, HCT-15 human colon cancer cells were knocked down for FPGS expression by RNA interference. The cell line stably expressing FPGS shRNA (FPGS shRNA HCT-15) was cloned and transferred subcutaneously into nude mice fed a low-folate diet. FPGS shRNA HCT-15 tumors expressed a significantly lower level of Fpgs at protein and mRNA levels than parental HCT-15 cells, and the levels of reduced folate in Fpgs shRNA HCT-15 tumors became 57% of those in parent after a single administration of 10 mg/kg of LV. Notably, FPGS downregulation did not affect the tumor growth or sensitivity to fluoropyrimidine. Importantly, we observed that LV given for 14 days failed to enhance the anticancer effects of UFT and S-1 in FPGS shRNA HCT-15. This was in keeping with the results that LV did not increase the ternary complex of TS, FdUMP and reduced folate. In conclusion, the present results provide *in vivo* evidence that intratumor FPGS plays an important role in the efficacy of oral fluoropyrimidine plus LV therapy for colorectal cancer.

**Introduction**

Combined chemotherapy with 5-fluorouracil (5-FU) and leucovorin (LV) has been widely used to treat patients with colorectal cancer (CRC). In Japan, UFT, oral fluoropyrimidines instead of intravenous 5-FU, with LV (UFT/LV) have been also used based on evidence of equivalent efficacy to 5-FU/LV for the treatment of CRC patients in both advanced and adjuvant settings (1-3). UFT is an orally active combination of tegafur (FT; a prodrug of 5-FU) and uracil [a competitive inhibitor of dihydropyrimidine dehydrogenase (DPD) that degrades 5-FU] and uracil [a potent inhibitor of dihydropyrimidine dehydrogenase (DPD) that degrades 5-FU] in a 1:4 molar ratio. In addition, S-1 is an orally active combined formulation of FT, gimeracil (CDHP; a potent inhibitor of DPD), and oteracil (Oxo; an inhibitor of orotate phosphoribosyltransferase (OPRT) that phosphorylates 5-FU in the gastrointestinal tract, which thereby reduces its toxic gastrointestinal effects) at molar ratios of 1:0.4:1 (4). The efficacy of S-1 for the treatment of metastatic CRC has been demonstrated in clinical studies (5), and phase II clinical trials of S-1/LV combination therapy for CRC are in progress in Asia.

Fluoropyrimidine derivatives have been shown to exert anticancer effects primarily through the inhibition of thymidylate synthase (TS) by forming covalent ternary complexes with FdUMP and a representative reduced folate, 5,10-methylenetetrahydrofolate (CH$_2$FH$_4$). Although LV itself has no antitumor activity, LV enhances the anticancer effect of 5-FU by increasing the intratumor levels of reduced folate, which resultantly stabilizing the ternary complex (6).

Many studies have discussed the factors that determine the clinical efficacy of 5-FU-based chemotherapy (6-9). However, limited information is still available with regard to the determinant of the LV effect. It is note that orally or intravenously administrated LV is metabolized to monoglutamate 5-(CH$_2$FH$_4$)$_2$, circulating form in blood, which is transported across the cell membrane. Once taken up into cells, intracellular folates are converted to polyglutamates by folylpolyglutamyl synthetase (FPGS), and polyglutamated folates are better retained in cells and more stabilize the ternary complex more than monoglutamate folates (10). FPGS has been shown to be one of the determinants of intracellular reduced folate level after LV treatment *in vitro* (11) and in clinical studies (12). However, it remains unclear *in vivo* or in clinical studies whether the FPGS status in the tumors affects the ability of LV to enhance the antitumor

**Key words:** folylpolyglutamate synthase, 5-fluorouracil, leucovorin, S-1, UFT
activity of fluoropyrimidine. Thus, using short hairpin RNA (shRNA) to downregulate FPGS, we assessed the impact of FPGS expression levels on LV-mediated enhancement of the intratumor reduced folate and the antitumor effects of UFT or S-1 in a mouse xenograft model.

Materials and methods

Materials. The FT, CDHP, Oxo, Uracil and LV used in this study were provided by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). Hydroxypropylmethylcellulose (HPMC) was purchased from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). [6-3H]-FdUMP and [6-14C]-5-FU were purchased from Moravek Biochemicals Inc. (Brea, CA, USA).

Cell lines and animals. The human colon cancer cell line HCT-15 was obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and was grown in RPMI-1640 medium (Sigma Aldrich Japan, Tokyo, Japan) with 10% fetal bovine serum (MP Biomedicals, Inc., Aurora, OH, USA). Male BALB/cA-nu/nu mice (5-week-old) were purchased from Charles River Japan Inc. (Tokyo, Japan). They were fed a low-folate pellet diet (AIN-93M-based Folate-Deficient Rodent Diet, Oriental Yeast Co., Ltd., Tokyo, Japan) with filtered water ad libitum. The low-folate diet contained 10% of the folate of the normal diet. The mice were kept in laminar air-flow units throughout the experiments, all of which were performed in compliance with the regulations of the Animal Experimentation Committee of Taiho Pharmaceutical Co., Ltd.

Generation of colon cancer cell lines stably expressing FPGS shRNA. The shRNA oligonucleotides for FPGS were purchased from Invitrogen (Gaithersburg, MD, USA). The shRNA oligonucleotides were subcloned into pBasik-hU6Neo vector (Takara, Shiga, Japan) and transfected into HCT-15 cells using Lipofectamine 2000 (Invitrogen). These cells were incubated with 700 µg/ml of g418 (gibco, Tokyo, Japan) with 10% fetal bovine serum (MP Biomedicals, Inc., Tokyo, Japan) and was grown in rpMI-1640 medium (sigma, St. Louis, MO, USA). The gene-expression products (Applied biosystems) and TaqMan probes were prepared using Assay-on-Demand Universal pcr Master mix (Applied biosystems, Foster city, CA, USa) using a TaqMan rt-pcr was performed on a prIsM 7900 sequence detector (Applied Biosystems, Foster City, CA, USA). The primers and TaqMan probes were prepared using Assay-on-Demand gene-expression products (Applied Biosystems).

Drug preparation and administration. UFT was prepared by mixing FT and Uracil at molar ratios of 1:4 in 0.5% HPMC solution. S-1 was prepared by mixing FT, CDHP and Oxo at molar ratios of 1:0.4:1 in 0.5% HPMC solution. The dosages of UFT or S-1 were expressed in terms of their contents of FT. LV was suspended in 0.5% HPMC solution. S-1 (8.3 mg/kg/day), UFT (16.7 mg/kg/day) and LV (10 mg/kg/day) were orally administered for 14 days.

Antitumor experiments. Human tumor xenografts were prepared by subcutaneous implantation of a tumor fragment (~2 mm in diameter) into the right side of the back of each nude mouse (at ~7-week-old). On 100-200 mm³ tumor volumes (TVs), the animals were randomized to control or treatment groups in such a way as to make the average TVs the same for the groups. The drugs were given for 14 consecutive days. Tumor size and body weight were measured twice weekly throughout the experiments. The TV, relative tumor volume (RTV), tumor growth-inhibition (TGI) rate and body-weight change (BWC) were calculated as follows:

TV (mm³) = length (mm) × width (mm) × width (mm) × 0.5

RTV = (TV on day 15)/(TV on day 0)

TGI (%) = [1 - (mean RTV of treatment group)/(mean RTV of control group)] × 100

BWC (%) = [(mean body weight at day 15)(mean body weight at day 0) - 1] × 100

Determination of TS activity. TS activity was determined as the number of [6-3H]-FdUMP binding sites in the 105,000 g supernatant of tumor tissue homogenates as described by Spears et al (13). An aliquot of the supernatant was used to determine the soluble protein by the method of Bradford. The supernatant was incubated with [6-3H]-FdUMP in the presence of excess CH₂FH₂ for 20 min at 30°C, and the radioactivity in the acid-insoluble fraction was measured with a liquid scintillation counter (TRI-CARB 2000CA; Packard Instruments, Meriden, CT, USA).

Determination of OPRT activity. Tumors were placed in 3-time volumes of ice-cold 10 mM Tris-HCl buffer (pH 7.4), containing 1 mM EDTA and 0.5 mM DTT and homogenized. OPRT activity was determined using a previously described 5-FU phosphorylation assay (14). The supernatant of tumor tissue homogenates was incubated with 20 µM [6-14C]-5-FU; 50 mM Tris-HCl, pH 8.0; 5 mM MgCl₂; 10 mM NaF; 0.5 mM DTT and 4 mM phosphoribosylpyrophosphate at 37°C for 10 min. The incubation was terminated by the addition of methanol followed by centrifugation at 10000 revolutions per min (rpm) for 5 min. The supernatant was dried up, and dissolved in distilled water (100 µl), and 40 µl aliquots were applied to the ODS column (Daiso, Osaka, Japan) in HPLC (Shimadzu, Kyoto, Japan). The mobile phase was used as follows: solvent A, acetonitrile; solvent B, 10 mM potassium dihydrogenphosphate buffer. Elution gradient used: the

\[ \text{bwc} = \frac{(\text{mean body weight at day 15})/ (\text{mean body weight at day 0}) - 1}{100} \]

Determination of intratumoral reduced folate level. The assay for reduced folate is based on the entrapment of CH₂FH₂ into a stable ternary complex with excess recombiant human TS protein and excess [6-3H]-FdUMP (15). Since CH₂FH₂ can potentially be dissociated into FH₂ and formaldehyde under these conditions, the sum of these folates is measured. Tumor tissues were homogenized with 3-time volumes of ice-cold...
10 mM phosphate buffer (pH 7.0), containing 2 mg/ml ascorbic acid and 40 mM 2-mercaptoethanol. After centrifugation, the supernatant was diluted with homogenizing buffer to 8 mg protein/ml, then placed immediately in a boiling water bath for 1 min and centrifuged to remove the precipitated protein. The supernatant was used for folate determination in a reaction mixture containing 5 µg recombinant human ts protein, 125 nM [6-3H]-FdUMP, and 6.5 mM formaldehyde in 200 µl of 50 mM tris-HCl buffer (pH 7.4). The supernatant (50 µl) was added to 200 µl of the reaction mixture, incubated at 30˚C for 50 min, and then the radioactivity in the acid-insoluble fraction was measured with a liquid scintillation counter.

Western blotting. Samples of lysates containing 40 µg protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, blocked in blocking solution (UK-B80, DS Pharma Biomedical Co., Ltd., Osaka, Japan), and incubated with primary antibodies (anti-human FPGS antibody diluted at 5 µg recombinant human TS protein, 125 nM [6-3H]-FdUMP, and 6.5 mM formaldehyde in 200 µl of 50 mM Tris-HCl buffer (pH 7.4). The supernatant (50 µl) was added to 200 µl of the reaction mixture, incubated at 30˚C for 50 min, and then the radioactivity in the acid-insoluble fraction was measured with a liquid scintillation counter.

Results

Establishment of colon cancer cell lines stably expressing FPGS shRNA. We first planned to establish the human colon cancer cell lines that express downregulated levels of Fpgs but keep the sensitivity to 5-FU in vitro compared to the parental cell lines. Several CRC cell lines were tested if they express significant levels of FPGS and respond to 5-FU in vitro. Selected cell lines were transfected with shRNA oligonucleotide for Fpgs, subcloned based on their expression of Fpgs shRNA. Cell lines stably expressed FPGS shRNA were subcloned and transferred subcutaneously into nude mice fed low folate diet (folate-deficient mice). Fig. 1 shows characteristic features of a representative cell line, the Hct-15 Fpgs shRNA; as compared with parental Hct-15 cells, they expressed low levels of Fpgs mRNA and protein in vitro and significantly lower levels of FPGS protein in vivo. Note that the intratumoral activities of TS and OPRT, which were reported to play an important role in the chemosensitivity of cancer cells to 5-FU (6-9), were invariably under FPGS shRNA (Table I).

Effect of FPGS downregulation on reduced folate levels in colon tumor xenografts. We next investigated the significance of FPGS in regulating intratumor reduced folate levels. Fig. 2 shows the levels of reduced folate in the tumors at 24 h after three 10-min washes, the membranes were incubated with enhanced ECL reagent (Thermo, Rockford, IL, USA) for 1 min and chemiluminescence was detected with LAS 3000 (Fujifilm, Tokyo, Japan).

Statistical analysis. The significance of differences in reduced folate level and RTV were analyzed with Student’s t-test and Aspin-Welch’s t-test respectively using EXSAS (version 7.11, Arm Co., Ltd., Osaka, Japan). p<0.05 was considered to be significant.
In vivo evidence for folylpolyglutamate synthase in combined chemotherapy

Vehicle or LV treatment. In control group, the levels of reduced folate in FPGS shRNA HCT-15 tumors were 66% of that in parent tumors. Single administration of LV significantly elevated the reduced folate levels in tumors, while the reduced folate levels in FPGS shRNA HCT-15 tumors became 57% of that in parent tumors in the LV treatment group (p<0.05).

Effect of FPGS downregulation on the antitumor effects of UFT or S-1 in combination with LV. Subsequently, we examined whether expression levels of FPGS affected the effectiveness of LV on antitumor activity of oral fluoropyrimidine with LV. UFT/LV were orally administered for 14 consecutive days to nude mice bearing parental HCT-15 or FPGS shRNA HCT-15 tumors (n=7). The tumor growth of FPGS shRNA HCT-15 was similar to that of parental HCT-15. LV alone had no antitumor activity, while UFT alone or UFT/LV treatment inhibited tumor growth regardless of FPGS downregulation (p<0.05; Fig. 3A). UFT/LV clearly inhibited parental HCT-15 tumor growth more effectively than UFT alone, however LV co-administration failed to further potentiate the antitumor effect of UFT in FPGS shRNA HCT-15 xenografts (Fig. 3A). On the other hand, LV co-administration resulted in body weight loss in the same manner in both parental HCT-15 and FPGS shRNA HCT-15 bearing mice, although this effect was tolerable. Furthermore, similar results were also observed in the case of the administration of S-1 instead of UFT (Fig. 3B), suggesting that FPGS downregulation suppressed LV-induced enhancement of antitumor effect of UFT or S-1 as a result of the loss of reduced folate induced by LV.

Table I. Effects of FPGS shRNA on TS and OPRT enzyme activities in tumor tissues of the human colon cancer xenografts.a

<table>
<thead>
<tr>
<th>Tumors</th>
<th>TS (pmol/mg protein)</th>
<th>OPRT (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>0.82±0.22</td>
<td>7.57±7.40</td>
</tr>
<tr>
<td>FPGS shRNA</td>
<td>0.66±0.01</td>
<td>6.52±3.70</td>
</tr>
</tbody>
</table>

a The TS and OPRT activities in parental HCT-15 or FPGS shRNA HCT-15 tumors were measured by the methods described in Materials and methods. TS activity was determined as the number of [6-3H]-FdUMP binding sites and OPRT activity was determined using 5-FU as a substrate. Values indicate the mean ± SD of 3 mice.
Effect of FPGS downregulation on the ternary complex formation. To support the data of antitumor activities, we measured the TS ternary complexes, thought to indicate the effect of 5-FU, using Western blotting in parental and FPGS shRNA HCT-15 tumor xenografts 24 h after treatment with UFT alone or UFT/LV for 14 consecutive days (Fig. 4). LV co-administration increased the ternary complex formation in parental HCT-15 tumors, however, the increase was not observed in FPGS shRNA HCT-15 tumors.

Discussion

The present study was designed to demonstrate the role of FPGS in potency of oral fluoropyrimidine combined with LV in vivo. To this end, we newly established the human colon cancer cell lines which stably expressed FPGS shRNA. Representative FPGS shRNA HCT-15 cells in culture expressed significantly lower levels of FPGS at mRNA and protein levels compared to parental HCT-15 cells. More importantly, HCT-15 FPGS shRNA tumor tissue isolated from nude mice retained the suppressed levels of FPGS expression at protein levels. It should be noted that FPGS downregulation exhibited no significant impact on the tumor growth or intra-tumoral activities of TS and OPRT, which may affect the efficacy of 5-FU treatment. The results were consistent with the findings reported by Sakamoto et al who showed no significant difference in the IC50 values of fluorodeoxyuridine between control siRNA and FPGS siRNA transfected cells transiently (11).

Based on these findings, it is reasonable to assume that the mouse xenograft model of downregulated FPGS is valid to investigate the role of FPGS in LV therapy in terms of intratumoral reduced folate levels as well as anticancer activities of UFT or S-1 plus LV. Therefore, we compare the changes in the levels of reduced folate in parental and FPGS shRNA HCT-15 cells before and after oral administration of LV. There was no significant difference in reduced folate levels between parental and FPGS shRNA HCT-15 cells, but an increase in intratumoral reduced folate after LV treatment was definitely suppressed in FPGS shRNA HCT-15 cells compared to those in parental cells. The results suggest that FPGS stabilize newly incorporated into cells more effectively than basal one, but further analysis is needed to understand the precise mechanism of intratumoral FPGS. Important is the observation that in contrast to those seen in parental cells, LV treatment failed to enhance the anticancer activity of UFT or S-1 combined with LV, in FPGS shRNA HCT-15 cells. Moreover, LV co-administration formed a much larger ternary complex than UFT alone in parental but not in FPGS shRNA HCT-15 cells. These data are in agreement with previous studies in that the increased levels of intratumoral reduced folate after LV treatment stabilized the ternary complex and potentiated the TS inhibition (16-18).

In conclusion, our study provides first in vivo evidence that intratumoral FPGS status may regulates the LV-enhanced anticancer activity of UFT and S-1. Taken together with the recent study that FPGS expression and activity may correlate with the cytotoxicity of 5-FU with or without LV by in vitro studies (19,20), we propose the requirement of further clinical studies to identify FPGS as a significant enzyme for predicting the efficacy of LV in combination with oral fluoropyrimidine.

Acknowledgments

We thank Professor J. Patrick Barron, the Department of International Medical Communications of Tokyo Medical University for helpful advice and revision of the manuscript.

References


