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ORIGINAL ARTICLE

Molecular determinants of folate levels after leucovorin administration in colorectal cancer

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Abstract
Purpose Oral leucovorin (LV) is used with uracil/tegafur (UFT) in the treatment of colorectal cancer (CRC). In order to find the factors related to the efficacy of LV in enhancing the antitumour effect of UFT, we investigated the relationships between the reduced folate levels in the CRC tissue after LV administration and the gene-expression levels of folate-metabolizing enzymes and folate transporters.
Methods The subjects were 60 CRC patients, scheduled to undergo surgery. The control group ($n = 30$) did not receive LV. Three groups ($n = 10$ for each) received a single dose of oral LV at 25 mg, 4, 12 or 18 h before surgery (LV 4 h, LV 12 h or LV 18 h groups, respectively). The reduced folate levels in plasma and tissues were measured by high-performance liquid chromatography (HPLC) or a thymidylate synthase-FdUMP binding assay, respectively. The intratumoral expression levels of 34 genes were quantitatively evaluated with a real-time polymerase chain reaction (RT-PCR) assay.
Results The reduced folate levels persisted for a longer period of time in the CRC tissue than in the plasma after LV administration. A multivariate logistic regression analysis revealed that high folylpolyglutamate synthase (FPGS) gene expression, low γ-glutamyl hydrolase (GGH) gene expression and low ATP-binding cassette sub-family C, number 1 (ABCC1) gene expression in CRC tissues were predictive factors for a high reduced folate level after LV administration.
Conclusions The expression level of FPGS, GGH and ABCC1 in CRC tissues could predict the reduced folate level after LV administration, and these factors may determine the efficacy of LV treatment.

Keywords Colorectal cancer · Folate · FPGS · GGH · ABCC1 · Leucovorin

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Introduction

Intravenous 5-fluorouracil (5-FU) combined with leucovorin (LV) is a major chemotherapeutic regimen for colorectal cancer (CRC) in metastatic and adjuvant settings. Oral uracil/tegafur (UFT), a prodrug of 5-FU, plus LV has a comparable therapeutic efficacy to intravenous 5-FU plus LV [1–3].

5-FU mainly exerts its anticancer activity by inhibiting thymidylate synthase (TS) via the formation of a ternary complex of TS, 5,10-methylenetetrahydrofolate (5,10-CH_2THF) and 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), which is the active metabolite of 5-FU [4]. TS is a key enzyme in the synthesis of deoxythymidine nucleotides, and its inhibition is thought to deplete dTTP, resulting in the suppression of DNA synthesis. 5,10-CH_2THF is the reduced folate cofactor required for normal TS catalytic reactions.

LV (5-formyltetrahydrofolate; 5-CHO-THF) is the most stable reduced folate. LV itself has no antitumour activity, but enhances the antitumour activity of 5-FU by supplying 5,10-CH_2THF and stabilizing the ternary complex [4]. Although the LV metabolic sequence is complex, it has been suggested that 10-formyltetrahydrofolate (10-CHO-THF), 5,10-CH_2THF, and tetrahydrofolate (THF) are intermediate metabolites, and that 5-methyltetrahydrofolate (5-CH_3THF) is the terminal metabolite [5]. Several groups of investigators have monitored LV and 5-CH_3THF levels in plasma after LV administration using high-performance liquid chromatography (HPLC), and have used radioenzymatic methods to assess the combined pool of 5,10-CH_2THF and THF in tissues [6–11]. These studies provided conflict results on the tumour retention of reduced folates after administration of LV in mouse models. Some investigators found that elevated reduced folate levels in tumour tissues decline rapidly after intraperitoneal or intravenous administration of LV [7, 8]. However, we recently reported that the reduced folate levels in tumour tissues rapidly elevated and persisted for over 24 h following oral LV administration [12]. On the other hand, the tumour retention of reduced folate in human has not been investigated.

Although TS levels are known to affect the antitumour activities of 5-FU [4], the factors related to the efficacy of LV in enhancing 5-FU antitumour effects have not been elucidated. In order to predict the effectiveness of combined UFT and oral LV treatment accurately, it is important to establish biomarkers for these agents. Therefore, in the present study, we initially measured the levels of reduced folates in plasma (LV and 5-CH_3THF), tumour tissue and adjacent normal mucosa (combined pool of 5,10-CH_2THF and THF) from CRC patients who did or did not receive a single oral LV dose before surgery. We next investigated the relationships between the reduced folate levels and the gene-expression levels of folate-metabolizing enzymes and folate transporters in the CRC tissue. Moreover, we explored the predictive genes of reduced folate level after LV administration to determine the factors related to LV efficacy.

Patients and methods

Patients

Patients with clinical stage II/III colorectal adenocarcinoma on preoperative diagnosis were eligible if they met the following inclusion criteria: age between 20 and 80 years; Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; no previous chemotherapy or radiation therapy; adequate oral intake ability; adequate bone-marrow function (white blood-cell count >3,000 μL^{-1} but <12,000 μL^{-1}, neutrophil count >1,500 μL^{-1}, haemoglobin level >9.0 g/dL, and platelet count >100,000 μL^{-1}), hepatic function (total bilirubin <1.5 mg/dL, aspartate aminotransferase and alanine aminotransferase <100 IU/L) and renal function (serum creatinine <1.5 mg/dL). The patient characteristics are shown in Table 1. Six patients subsequently classified as pathological stage (pStage) I and four patients classified as pStage IV according to pathological diagnosis. All patients gave written informed consent. This study was approved by the ethics committees of Tokai University School of Medicine, Japan, and Taiho Pharmaceutical Co., Ltd (Tokyo, Japan).

Samples

In total, 60 patients were randomly assigned to the following four groups: a control group in which the patients did not receive LV ($n = 30$); and three other groups

Table 1 Patient characteristics

Characteristic	Control ($n = 30$) (%)	LV treatment			*P* value
		4 h ($n = 10$) (%)	12 h ($n = 10$) (%)	18 h ($n = 10$) (%)	
Age (years)					
Median	70	67	69	70	NS[a]
Range	45–93	36–83	49–90	54–93	
Gender					
Male	23 (77)	4 (40)	2 (20)	6 (60)	0.009[b]
Female	7 (23)	6 (60)	8 (80)	4 (40)	
pStage					
I	1 (3)	0	2 (20)	3 (30)	NS[b]
II	11 (37)	5 (50)	5 (50)	3 (30)	
III	17 (57)	5 (50)	2 (20)	2 (20)	
IV	1 (3)	0	1 (10)	2 (20)	
Histological type					
Well	14 (47)	4 (40)	6 (60)	7 (70)	NS[b]
Moderate	14 (47)	5 (50)	4 (40)	3 (30)	
Poor	1 (3)	0	0	0	
Mucious	1 (3)	1 (10)	0	0	
Lymph-node metastasis					
No	12 (40)	6 (60)	7 (70)	6 (60)	NS[b]
Yes	18 (60)	4 (40)	3 (30)	4 (40)	

The patients classified into pStageIV were found to have microscopic peritoneal metastasis during the pathological diagnosis performed after surgery

pStage pathological stage

[a] Not significant; Welch's *t* test

[b] Chi-square test

($n = 10$ for each) in which the patients received a single 25 mg dose of oral LV (Taiho Pharmaceutical Co., Ltd.) 4, 12 or 18 h before surgery. All patients fasted for 24 h before surgery. Peripheral blood was collected immediately before surgery. CRC tissue and adjacent normal mucosa were obtained from resected specimens within 10 min of excision. The specimens were divided into two, and one-half was immediately put into RNAlater (Ambion, Austin, TX, USA) according to the manufacturer's protocol for gene-expression analysis, whereas the other half was stored at −80°C for measurement of reduced folate.

Measurement of LV and 5-CH_3THF plasma levels

The plasma LV and 5-CH_3THF levels were determined by the validated HPLC method [9]. Briefly, LV and 5-CH_3THF were extracted from the plasma as previously described [13]. LV was resolved from endogenous interference by gradient HPLC (mobile phase A, 40% acetonitrile–50% methanol in 25 mmol/L KH_2PO_4, pH 2.3; mobile phase B, 25 mmol/L KH_2PO_4, pH 2.3), and 5-CH_3THF was resolved by isocratic HPLC with a mobile phase consisting of 5% acetonitrile–5% methanol in 25 mmol/L KH_2PO_4, pH 2.3. Both LV and 5-CH_3THF were detected at 310 nm [14]. Levels under the lower limit of quantitation were assigned a value of zero for the analysis.

Measurement of reduced folate levels in tissues

The tissue folate levels were measured as the combined pools of 5,10-CH_2THF and THF, using the TS-FdUMP binding assay [5]. Tissues were homogenized with three volumes of ice-cold 10 mmol/L phosphate buffer (pH 7.0) containing 2 mg/mL ascorbic acid and 40 mmol/L 2-mercaptoethanol. After centrifugation, the protein contents in the supernatants were measured using a BioRad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The supernatants were boiled for 1 min and centrifuged to remove precipitated protein. The supernatant (50 μL) was incubated in 200 μL 50 mmol/L Tris–HCl buffer (pH 7.4) containing 5 μg recombinant human TS protein (Taiho Pharmaceutical Co., Ltd.), 125 nmol/L [6-^{3}H] FdUMP (555 GBq/mmol) (Moravek Biochemicals Inc., Brea, CA, USA) and 6.5 mmol/L formaldehyde at 30°C for 50 min. The radioactivity in the acid-insoluble fraction was measured with a liquid-scintillation counter (TRI-CARB 2000CA; Packard Instruments, Meriden, CT, USA). Levels under the lower limit of quantitation were assigned a value of zero for the analysis.

RNA isolation and quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the tissue using the RNeasy mini kit (Qiagen, Tokyo, Japan) and reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Real-time RT-PCR was performed on the ABI PRISM 7900HT sequence detection system using a real-time RT-PCR array (Taqman array; Applied Biosystems), which included duplicated wells of six reference genes and 28 target genes (Table 2), according to the manufacturer's protocol. The gene-expression levels were normalized to the geometric mean of the reference genes [actin, beta (ACTB), beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein, large, P0 (*RPLP0*), ubiquitin C (UBC) and ribosomal protein L13 (*RPL13*).

Statistical analysis and clustering

For clustering analysis, the gene-expression levels were normalized to the median expression level. Clustering was performed using GeneSpring software (Agilent Technologies, Santa Clara, CA, USA) and the standard correlation. Dunnett's multiple test, Welch's t test and the Chi-square test were used to analyse the differences between groups. Pearson's correlation was used to estimate the degree of association between the reduced folate level and the $\log_2$-transformed gene-expression level. A multivariate logistic regression model with a stepwise regression was used to assess the independent effect of gene-expression level on the reduced folate level after LV administration. For the logistic regression analysis, the reduced folate level and the expression levels of 28 target genes in CRC tissues after LV administration were dichotomized into two classes: values below or equal to the median value of 30 patients treated with LV and values above the median value. Probabilities to enter and remove variables were settled at less than 0.05. JMP software (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. Data were considered statistically significant at $P < 0.05$.

Results

Plasma concentrations of LV and 5-CH_3THF

Plasma LV and 5-CH_3THF were not detected in the control group. The plasma LV levels in the LV 4 h, LV 12 h and LV 18 h groups were 325.5 ± 181.8, 87.6 ± 47.3 and 76.9 ± 60.2 pmol/mL, respectively (Fig. 1a). Compared

Table 2 Gene list

Category	Gene symbol	Gene name	Assay ID
Reference gene	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
	ACTB	Actin, beta	Hs99999903_m1
	RPLP0	Ribosomal protein, large, P0	Hs99999902_m1
	UBC	Ubiquitin C	Hs00824723_m1
	RPL13	Ribosomal protein L13	Hs00742932_s1
	B2M	Beta-2-microglobulin	Hs99999907_m1
Folate metabolism	ALDH1L1	Aldehyde dehydrogenase 1 family, member L1	Hs00201836_m1
	AMT	Aminomethyltransferase (glycine cleavage system protein T)	Hs00166628_m1
	ATIC	5-Aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	Hs00269671_m1
	DHFR	Dihydrofolate reductase	Hs00758822_s1
	DMGDH	Dimethylglycine dehydrogenase	Hs00203638_m1
	FOLH1	Folate hydrolase (prostate-specific membrane antigen) 1	Hs00379515_m1
	FPGS	Folylpolyglutamate synthase	Hs00191956_m1
	GART	Phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	Hs00531926_m1
	GGH	Gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	Hs00608257_m1
	MTFMT	Mitochondrial methionyl-tRNA formyltransferase	Hs00373739_m1
	MTHFD1	Methylenetetrahydrofolate dehydrogenase (NADP + dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	Hs00602830_m1
	MTHFD1L	Methylenetetrahydrofolate dehydrogenase (NADP + dependent) 1-like	Hs00383616_m1
	MTHFD2	Methylenetetrahydrofolate dehydrogenase (NADP + dependent) 2, methenyltetrahydrofolate cyclohydrolase	Hs00759197_s1
	MTHFR	5,10-Methylenetetrahydrofolate reductase (NADPH)	Hs00195560_m1
	MTHFS	5,10-Methenyltetrahydrofolate synthetase (5-formyltetrahydrofolate cyclo-ligase)	Hs00197574_m1
	MTR	5-Methyltetrahydrofolate-homocysteine methyltransferase	Hs00165188_m1
	MTRR	5-Methyltetrahydrofolate-homocysteine methyltransferase reductase	Hs00242867_m1
	SHMT1	Serine hydroxymethyltransferase 1 (soluble)	Hs00541038_m1
	SHMT2	Serine hydroxymethyltransferase 2 (mitochondrial)	Hs00193658_m1
Folate transporters	ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	Hs00219905_m1
	ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	Hs00166123_m1
	ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	Hs00358656_m1
	ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	Hs00195260_m1
	ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	Hs00184979_m1
	FOLR1	Folate receptor 1 (adult)	Hs00357143_g1
	FOLR2	Folate receptor 2 (foetal)	Hs00265255_m1
	FOLR3	Folate receptor 3 (gamma)	Hs00357145_g1
	SLC19A1	Solute carrier family 19 (folate transporter), member 1	Hs00953344_m1

with the LV 4 h group, the plasma LV levels in the LV 12 h and LV 18 h groups were reduced to 27 and 24%, respectively (Fig. 1a). The plasma 5-CH_3THF levels in the LV 4 h, LV 12 h and LV 18 h groups were 224.1 ± 63.8, 27.2 ± 35.7 and 9.0 ± 28.6 pmol/mL, respectively (Fig. 1b). LV administration significantly increased the plasma LV levels in all three LV groups, but significantly increased the 5-CH_3THF levels only in the LV 4 h group compared with the control group.

Reduced folate levels in CRC tissue and adjacent normal mucosa

The tissue levels of the combined pool of 5,10-CH_2THF and THF are determined. In the control group, the reduced folate levels in the CRC tissue and adjacent normal mucosa were 2.00 ± 2.14 and 1.94 ± 1.24 pmol/mg protein, respectively, which were not significantly different (Welch's t test $P = 0.89$).

The reduced folate levels in the adjacent normal mucosa derived from the LV 4 h, LV 12 h and LV 18 h groups were 3.27 ± 1.20, 3.60 ± 1.10 and 3.34 ± 1.28 pmol/mL, respectively (Fig. 1c). LV administration significantly increased the reduced folate levels in the adjacent normal mucosa compared with the control group, and the increase continued until 18 h after the LV treatment. The reduced folate levels in the CRC tissue derived from the LV 4 h, LV 12 h and LV 18 h groups were 2.48 ± 3.48, 4.88 ± 4.51 and 3.28 ± 2.95 pmol/mL, respectively (Fig. 1d). LV administration also increased the reduced folate levels in the CRC tissue, and a significant difference was observed between the LV 12 h group and the control group.

The reduced folate levels in the CRC tissue after LV administration varied more widely than in the adjacent normal mucosa.

Gene-expression profiling in CRC tissue and adjacent normal mucosa

The expression levels of 28 genes encoding folate-metabolizing enzymes and folate transporters in the CRC tissue and adjacent normal mucosa were detected by a real-time RT-PCR array. Unsupervised hierarchal clustering analysis of tissues from all 60 CRC patients identified two groups: one consisting mostly of CRC tissue, and one consisting mostly of adjacent normal mucosa (Fig. 2). There was no correlation between the cluster groups with or without LV treatment (data not shown).

Associations between reduced folate levels and gene-expression levels in CRC tissue

Table 3 shows the genes that were correlated with the reduced folate levels in the CRC tissue in both the control group and the LV-treated group (the LV 4 h, LV 12 h and LV 18 h groups combined). In the control group, the expression levels of genes encoding the folate-metabolizing enzymes 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC), dihydrofolate reductase (DHFR) and γ-glutamyl hydrolase (GGH) were negatively correlated with the reduced folate levels. In the LV-treated group, the expression levels of genes encoding the folate transporter ATP-binding cassette sub-family C, number 1 (ABCC1) and the folate-metabolizing enzyme folate hydrolase 1 (FOLH1) were negatively correlated with the reduced folate levels, whereas a gene encoding the folate-metabolizing enzyme folylpolyglutamate synthase (FPGS) was positively correlated with the reduced folate levels.

Moreover, we performed a multivariate logistic regression analysis to identify genes capable of predicting reduced folate levels after LV administration. The reduced

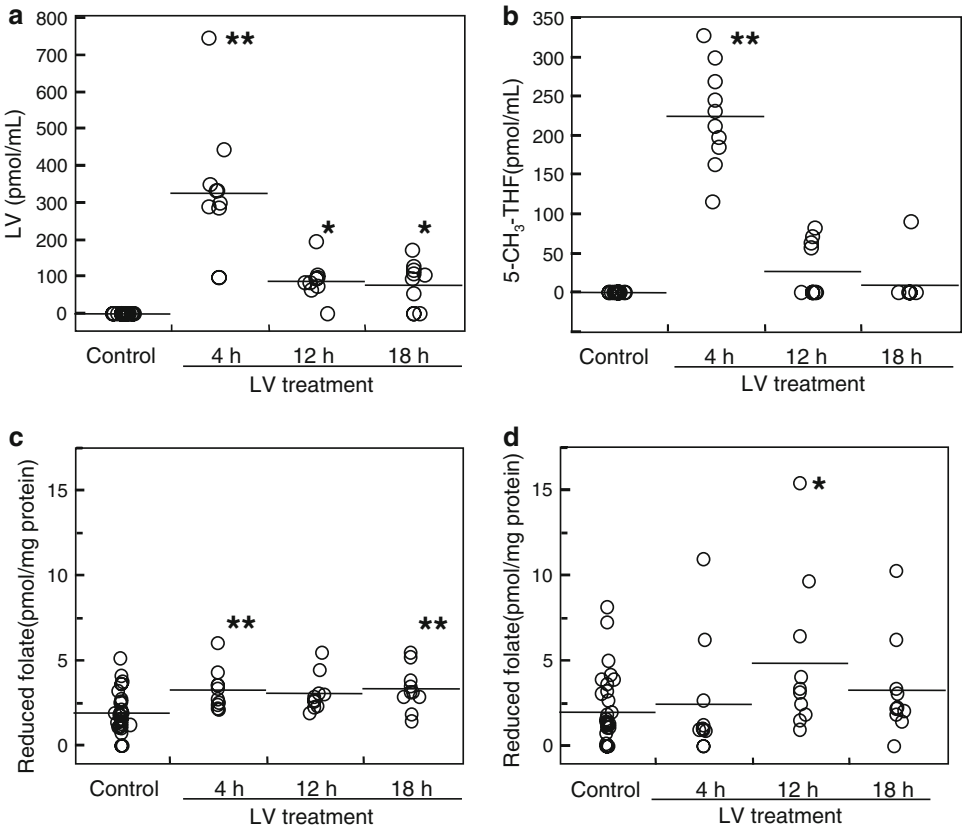

Fig. 1 LV (**a**) and 5-CH_3-THF (**b**) levels in plasma and reduced folate levels in adjacent normal tissue (**c**) and CRC tissues (**d**) derived from control and LV-treated CRC patients. The *horizontal line* represents the mean values. $^*P < 0.05$, $^{**}P < 0.01$ compared with the control group (Dunnett's multiple test)

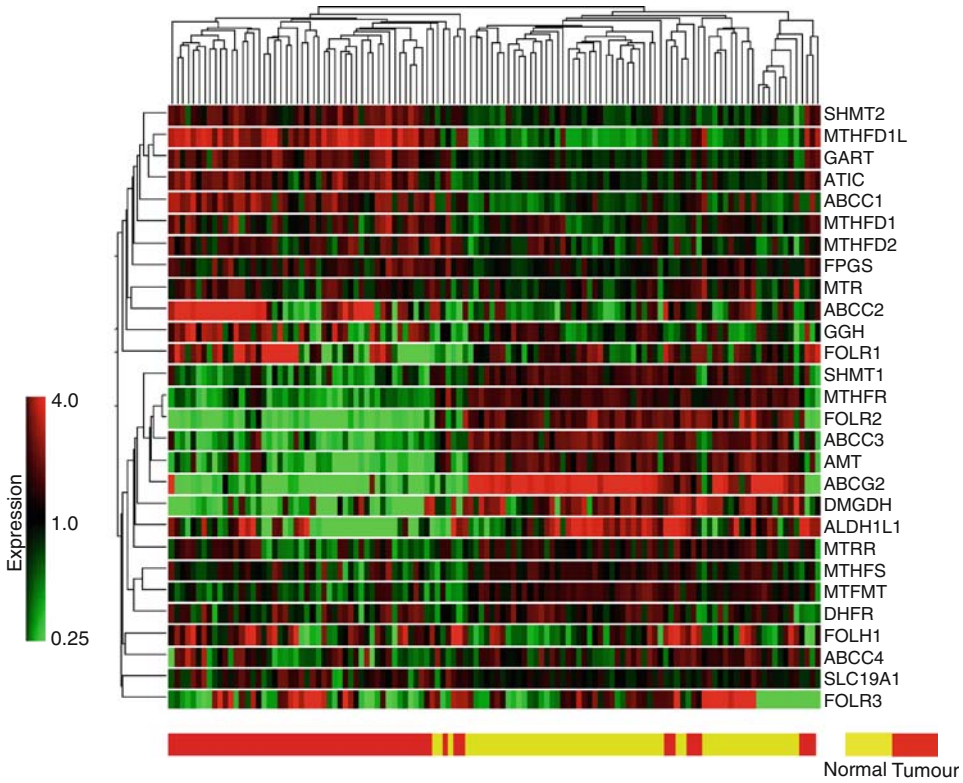

Fig. 2 Gene-expression profiling in CRC tissue and adjacent normal mucosa. Expression levels of 28 genes encoding enzymes of folate metabolism and transporters of folate in CRC tissue and adjacent normal mucosa derived from 60 CRC patients

folate level and the expression of genes in CRC tissues-derived LV-treated group were dichotomized into two classes: values below or equal to the median value of 30 patients and values above the median value. When variables were selected using stepwise regression, the odds ratios for a high level of reduced folate were 0.050 for low GGH gene expression (95% confidence interval [CI]), 0.0020–0.44; $P = 0.020$), 0.089 for low ABCC1 gene expression (95% CI, 0.0073–0.65; $P = 0.029$), and 14.35 for high FPGS gene expression (95% CI, 1.6–369; $P = 0.041$) (Table 4). When these three genes were used to predict the level (high or low) of reduced folate, the accuracy was 83.3% (25 out of 30 patients), with a sensitivity of 86.7% and a specificity of 80.0%. Thus, the gene expressions of GGH, ABCC1 and FPGS served as important independent variables for predicting the reduced folate level after LV administration.

Furthermore, the expression levels of these GGH, ABCC1 and FPGS in CRC tissues did not differ between the control group and the LV-treated group (Welch's t test $P = 0.22$, $P = 0.63$, $P = 0.92$, respectively).

Discussion

Although the pharmacokinetics of LV in human blood has previously been investigated, the LV pharmacokinetics in tumour tissues have only been examined in mouse models [5, 11, 15, 19]. Recently, Schlemmer et al. [11] reported reduced folate tissue levels in CRC patients after intravenous LV infusion; however, they did not show tumour retention of reduced folates. To our knowledge, the current study is the first to analyse the reduced folate levels over time in the plasma and tissues of CRC patients. We found that these levels were significantly increased after the oral administration of LV. We also demonstrated that the reduced folate levels in plasma decreased rapidly with time, whereas the reduced folate levels in the CRC tissue and adjacent normal mucosa tended to be retained for longer periods. These results indicate that tissues have an ability to retain the reduced folate for longer period following the oral administration of LV, and this finding could be useful for the development of therapeutic strategies.

Gene-expression profiling revealed different expression patterns for genes encoding folate-metabolizing enzymes and folate transporters in the CRC tissue compared with the adjacent normal mucosa. This suggests that the regulation of folate metabolism in CRC tissue differs from that in normal mucosa. The reduced folate levels in the CRC tissue varied more widely than those in the adjacent normal mucosa after LV treatment. This supports the finding of Schlemmer et al., who showed inter-individual variation of reduced folates in CRC patient tissues after intravenous LV infusion [11]. Moreover, Houghton et al. [6] reported that the increase in the level of the reduced folate after infusion LV differed among four human CRC xenografts. These

Table 3 Correlation analysis between reduced folate level and the gene-expression level in CRC tissue

Gene symbol	Gene name	*r*	*P* value
Control group			
ATIC	5-Aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	–0.42	0.022
DHFR	Dihydrofolate reductase	–0.42	0.022
GGH	Gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	–0.38	0.037
LV-treated group			
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	–0.45	0.013
FPGS	Folylpolyglutamate synthase	0.43	0.018
FOLH1	Folate hydrolase (prostate-specific membrane antigen) 1	–0.40	0.028

r Pearson's correlation coefficient

Table 4 Multivariate logistic regression analysis for reduced folate level after LV administration in CRC tissue

Gene symbol	Estimate	Odds ratio	95% CI	*P* value
GGH	−2.99	0.050	(0.0020–0.44)	0.020
ABCC1	−2.42	0.089	(0.0073–0.65)	0.029
FPGS	2.66	14	(1.6–369)	0.041

95% *CI* 95% confidence interval

results suggest that inter-individual variations in the regulation of folate metabolism in CRC tissues likely exist and that these variations might be responsible for the differences in the reduced folate levels after LV administration. Thus, the effectiveness of LV in enhancing the antitumour activity of 5-FU might differ among CRC patients.

High FPGS gene expression, low GGH gene expression and low ABCC1 gene expression in CRC tissues after LV administration were selected as predictive factors of a high reduced folate level after LV administration using a multivariate logistic regression analysis. Furthermore, the expression levels of these GGH, ABCC1 and FPGS in CRC tissues did not differ between the control group and the LV-treated group. These results indicate that a single dose of oral LV did not affect the gene-expression levels of these three genes, suggesting that the gene-expression levels of FPGS, GGH and ABCC1 in CRC tissues before LV administration could be useful for predicting the reduced folate level after LV administration and might be useful for identifying of CRC patients who would benefit from LV therapy.

Circulating blood folate exists in a monoglutamate form, while intercellular folate exists primarily as polyglutamate form. Intercellular folate is converted into an polyglutamate form by FPGS, which catalyzes the sequential addition of glutamic acid to reduced folate derivatives. GGH is a lysosomal enzyme that acts as an endo-peptidase and/or exo-peptidase removes the terminal glutamates of the polyglutamated folates synthesized by FPGS [16, 17]. The polyglutamate forms of folate are more strongly retained within the cell [16]. We reported previously that the small-interfering RNA (siRNA) downregulation of FPGS mRNA decreased both the intracellular folate level after LV treatment and the efficacy of LV in enhancing the antitumour effect of FdUrd in cultured human colon cancer cells. We also reported that the siRNA downregulation of GGH mRNA increased both the intracellular folate level after LV treatment and the cellular sensitivity to FdUrd plus LV [18]. In the present study, we found that the expression level of GGH was negatively related with the reduced folate levels in CRC tissue from control group, consistent with previous publications [19], and LV-treated groups. This study also found that the expression level of FPGS was positively correlated with the reduced folate levels in CRC tumour tissue from patients who received LV. These results indicate that the expression levels of FPGS and GGH are important for the regulation of the folate levels in tumour tissue from CRC patients after LV administration. The expression levels of these enzymes in tumour tissue might be closely related to the efficacy of LV in CRC patients.

ABCC1 is an ATP-binding cassette transporter, a member of the multiple-resistance protein (MRP) family, which mediates the cellular export of folate, including LV and antifolates such as methotrexate [20]. However, long-chain polyglutamate derivatives of folates and antifolates are not MRP substrates. Thus, ABCC1 might regulate the reduced folate levels in tumour tissue after the administration of LV by exporting monoglutamate or short-chain polyglutamate forms of the reduced folate, including LV.

Other folate-metabolizing enzymes such as ATIC, DHFR and FOLH might also be involved in the regulation of reduced folate level in CRC tissue. ATIC is a bifunctional enzyme that catalyzes the last two steps in the de

novo purine biosynthetic pathway. In the penultimate step, ATIC catalyzes the formylation of aminoimidazole-4-carboxamide ribonucleotide (AICAR) by 10-CHO-THF to produce 5-CHO-AICAR and THF [21]. DHFR catalyzes the conversion of dihydrofolic acid into THF, whereas FOLH1 hydrolyzes the γ-glutamyl tail linkage of folate polyglutamate, like GGH, although it is a transmembrane protein the active site of which is outside the cell [22]. Further studies will be required to clarify the relationship between folate-metabolizing enzymes and reduced folate levels in tumour tissues.

Our present findings suggest that the gene-expression levels of FPGS, GGH and ABCC1 in CRC tissues before LV administration could predict the reduced folate level after LV administration. There is a need to identify the relationships between these factors and the outcome of CRC patients receiving chemotherapy. To this end, we are currently investigating whether these factors are prognostic indicators in CRC patients undergoing oral UFT/LV chemotherapy.

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