Folic Acid-Metabolizing Enzymes Regulate the Antitumor Effect of 5-Fluoro-2′-Deoxyuridine in Colorectal Cancer Cell Lines

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Abstract

In colorectal cancer chemotherapy, the current standard of care includes combination therapy with 5-fluorouracil (5-FU) and leucovorin (LV). However, the factors that determine the LV-mediated enhancement of 5-FU antitumor activity are not fully understood. Therefore, we investigated the roles of thymidine synthase (TYMS), folate receptor 1 (FOLR1), dihydrofolate reductase (DHFR), phosphoribosylglycinamide formyltransferase (GART), methylenetetrahydrofolate dehydrogenase (MTHFD1), and methylenetetrahydrofolate reductase (MTHFR) in LV-mediated enhancement of 5-fluoro-2′-deoxyuridine (FdUrd) cytotoxicity in vitro as a model of 5-FU antitumor activity. These genes were downregulated in DLD-1 and HCT116 human colorectal cancer cells by using small-interfering RNA. Reduced expression of TYMS mRNA significantly increased FdUrd cytotoxicity by 100- and 8.3-fold in DLD-1 and HCT116 cells, respectively. In contrast, reducing the expression of FOLR1, DHFR, GART, MTHFD1, and MTHFR decreased FdUrd cytotoxicity by 2.13- to 12.91-fold in DLD-1 cells and by 3.52- to 10.36-fold in HCT116 cells. These results demonstrate that folate metabolism is important for the efficacy of FdUrd. Overall, the results indicate that it is important to clarify the relationship between folate metabolism-related molecules and 5-FU treatment in order to improve predictions of the effectiveness of 5-FU and LV combination therapy.

Introduction

Worldwide, colorectal cancer (CRC) was the third most common cancer (9.7%) and the fourth leading cause of cancer-related deaths in 2012 [1]. In CRC chemotherapy, the current standard of care includes combination treatment with 5-fluorouracil (5-FU) and leucovorin (LV). One of the anticancer mechanisms of 5-FU involves the inhibition of thymidylate synthase (TYMS) via formation of a ternary complex between TYMS, 5,10-methylenetetrahydrofolate (5,10-CH_{2}FH_{4}), and 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), which is the active form of 5-FU (Fig 1). Although LV itself has no antitumor activity, it has been used to increase the intracellular concentration of 5,10-CH_{2}FH_{4}, thereby increasing the efficacy of 5-FU.
LV enters cells via the reduced folate carrier, where it is metabolized to $5,10$-$\text{CH}_2\text{FH}_4$ and then polyglutamated by polyglutamate synthetase (FPGS) [2]. Polyglutamation not only increases the cellular retention of $5,10$-$\text{CH}_2\text{FH}_4$ but also enhances the stabilization of its ternary complex with TYMS and FdUMP [2]. The terminal glutamates of the polyglutamated folates synthesized by FPGS are removed by gamma-glutamyl hydrolase (GGH) [3]. Sakamoto et al. [4] found that in DLD-1 cells, suppression of FPGS by siRNA reduced the basal level of reduced folate and the folate level after LV treatment, and enhanced the ability of LV to increase FdUrd-induced cytotoxicity. Similarly, downregulation of GGH by siRNA increased the sensitivity of cells to 5-fluoro-2'-deoxyuridine (FdUrd) when combined with LV [4]. These results suggest that expression of both FPGS and GGH within tumor cells controls the ability of LV to enhance the antitumor activity of 5-FU by regulating folate levels.

Verifying the relationship between folic acid metabolism-related molecules and the efficacy of FdUrd in vitro is important to understand the contribution of folic acid metabolism to in vivo 5-FU-based chemotherapy. To investigate this relationship, we used siRNA to suppress the expression of the following folic acid metabolism-related genes: TYMS, folate receptor 1 (FOLR1), dihydrofolate reductase (DHFR), phosphoribosylglycinamide formyltransferase (GART), methylenetetrahydrofolate dehydrogenase 1 (MTHFD1), and methylene-tetrahydrofolate reductase (MTHFR). The effect of downregulating these genes on cell proliferation and the inhibitory effect of FdUrd were then evaluated using two different CRC cell lines in vitro.

Materials and Methods

Drugs

FdUrd was purchased from Tokyo Chemical Industry (Tokyo, Japan). Leucovorin was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell lines

Two human CRC cell lines (DLD-1 and HCT116) were obtained from the American Type Culture Collection (Manassas, VA, USA). DLD-1 was grown in Roswell Park Memorial Institute 1640 medium (Sigma–Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (Sigma–Aldrich Japan, Tokyo, Japan), and HCT116 was grown in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Osaka, Japan) with 10% fetal bovine serum.

Cytotoxicity test

Cells were seeded at a density of $2 \times 10^3$ (DLD-1) and $1 \times 10^3$ (HCT116) cells per well in 96-well plates, incubated overnight, and then treated with the drug for 72 h. Cell numbers were determined using a simplified crystal violet staining method [5]. IC$_{50}$ values, representing the respective concentrations at which 50% of cell growth was inhibited, were calculated from the regression line of concentration-response cytotoxicity plots as the concentration that showed 50% absorbance of the control using XLfit software (ID Business Solutions, Guildford, UK).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was performed on a PRISM$^\text{TM}$ 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) using TaqMan$^\text{TM}$ Universal PCR Master Mix (Applied Biosystems). Gene expression levels were normalized to those of $\beta$-actin (ACTB). The primers and TaqMan$^\text{TM}$
probes were prepared using Assay-on-Demand gene-expression products (Applied Biosystems) and Human ACTB Endogenous Control (VIC / MGB Probe, Applied Biosystems). Probe IDs were Hs00195560_m1 for MTHFR, Hs01068263_m1 for MTHFD1, Hs00894582_m1 for GART, Hs01124179_g1 for FOLR1, Hs00758822_s1 for DHFR, and Hs00426586_m1 for TYMS.

Silencing of folic acid metabolism-related genes

The siRNA oligonucleotides for MTHFR (ID: HSS106762) and MTHFD1 (ID: HSS106759) were prepared using Stealth RNAi™ (Thermo Fisher Scientific, Waltham, MA, USA). siRNAs for GART (ID: s735) and FOLR1 (ID: s5330) were prepared using Silencer® siRNA (Thermo Fisher Scientific). siRNA for DHFR (ID: SI00299992) was prepared using FlexiTube siRNA (Qiagen, Venlo, The Netherlands). siRNA for TYMS was prepared using MISSION® siRNA (Sigma-Aldrich). The sense and antisense strand sequences of TYMS siRNA were 5'-CAAUC CGCAUCCCAACUAUUTT -3' and 5'-AAUAGUUUGGAUGCGGA UUUGTT -3', respectively. The control siRNA oligonucleotide was Stealth RNAi™ Negative Control Med GC (Thermo Fisher Scientific). Cells were plated into 6-well plates at a density of 2.5 × 10^4 cells per well, incubated overnight, and then treated with RNAi duplex–Lipofectamine® RNAiMAX (Thermo Fisher Scientific) complexes (final concentration, 10 nM) for 24 h.

Statistical analyses

Statistical analyses were performed using Student’s t-test or Dunnett’s multiple test with JMP (SAS Institute Inc., Cary, NC, USA). P < 0.05 was considered statistically significant.
Results

Effect of downregulating TYMS on FdUrd cytotoxicity

To examine the influence of TYMS expression on the cytotoxicity of FdUrd, we abolished the expression of TYMS in DLD-1 and HCT116 cells using siRNA (S1 Table). Cells treated with control or TYMS siRNA were then exposed to increasing concentrations of FdUrd (0.003–30 μM) for 72 h, and survival relative to untreated cells was determined (Fig 2). The sensitivity of cells to FdUrd was increased in TYMS-downregulated cells, especially at low concentrations (0.003–0.3 μM). In control siRNA-treated DLD-1 and HCT116 cells, the IC$_{50}$ values of FdUrd were 0.67 and 0.25 μM, respectively. In DLD-1 and HCT116 cells treated with TYMS siRNA, the IC$_{50}$ values of FdUrd were 0.01 and 0.03 μM, respectively. The ratios of IC$_{50}$ values in cells treated with control and TYMS siRNA were 67 and 8.3 in DLD-1 and HCT116 cells, respectively (Table 1).

Effect of downregulating TYMS on the enhancement of FdUrd cytotoxicity by LV

We next examined the influence of TYMS on LV-enhanced FdUrd cytotoxicity. LV significantly enhanced FdUrd cytotoxicity in a concentration-dependent manner (0.1–10 μM) and at

Table 1. Change of sensitivity to FdUrd upon silencing of folic acid metabolism-related genes.

|          | Fold change of IC$_{50}$ versus control |
|----------|----------------------------------------|
|          | DLD-1 cells   | HCT116 cells  |
| TYMS     | 0.01          | 0.12          |
| FOLR1    | 12.9          | 10.4          |
| DHFR     | 6.18          | 6.80          |
| GART     | 8.03          | 9.84          |
| MTHFD1   | 4.07          | 7.96          |
| MTHFR    | 2.13          | 3.52          |

Comparison of IC$_{50}$ values in target siRNA-treated cells and control siRNA-treated cells.

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all concentrations in both DLD-1 and HCT116 cells treated with control siRNA (p < 0.01). The relative survival of cells treated with 0.03 μM FdUrd and 0.1, 1, or 10 μM LV was 86.6, 81.5, and 77.0% in DLD-1 cells, and 83.7, 75.1, and 72.1% in HCT116 cells, respectively. In contrast, LV did not enhance FdUrd cytotoxicity in \(TYMS\)-downregulated DLD-1 or HCT116 cells at any concentration (Fig 3).

**Effect of downregulating \(MTHFR\), \(MTHFD1\), \(DHFR\), \(GGH\), and \(GART\) on FdUrd cytotoxicity**

To investigate the influence of the supply of 5,10-CH\(_2\)-FH\(_4\) on FdUrd cytotoxicity, we downregulated folic acid-metabolizing enzymes and evaluated the sensitivity of DLD-1 and HCT116 cells to FdUrd. In DLD-1 cells, downregulation of folic acid-metabolizing enzymes, except for \(MTHFR\), reduced the efficacy of FdUrd. The IC\(_{50}\) values of cells with downregulated \(FOLR1\), \(DHFR\), \(GART\), and \(MTHFD1\) were 8.65, 4.14, 5.38, and 2.73 μM, respectively. The IC\(_{50}\) value of DLD-1 cells treated with control siRNA was 0.67 μM. In HCT116 cells, siRNA against \(FOLR1\), \(DHFR\), \(GART\), \(MTHFD1\), and \(MTHFR\) also reduced the sensitivity to FdUrd with IC\(_{50}\) values of 2.59, 1.70, 2.46, 1.99, and 0.88 μM, respectively, compared to the control siRNA IC\(_{50}\) value of 0.25 μM (Figs 4–8). The ratios of the changes of IC\(_{50}\) values in cells treated with each siRNA compared to the control siRNA treated cells were listed in Table 1.

**Discussion**

To investigate the influence of folic acid-metabolizing enzymes on the efficacy of FdUrd, the active form of 5-FU, we downregulated the corresponding genes by using siRNA. Suppression of \(TYMS\) enhanced the efficacy of FdUrd in human colorectal tumor cells. In contrast, suppression of \(GART\), \(DHFR\), \(MTHFD1\), and \(FOLR1\) in DLD-1 cells and \(GART\), \(DHFR\), \(MTHFD1\), \(FOLR1\), and \(MTHFR\) in HCT116 cells decreased the efficacy of FdUrd.

Balancing of the amounts of FdUMP, TYMS, and reduced folate in cells appears to be important for the inhibition of TYMS through the formation of a ternary complex [6]. Interestingly, the enhanced anti-proliferative effect of FdUrd after downregulation of TYMS was more remarkable at low concentrations (< 0.3 μM) than at high concentrations (>0.3 μM) in both HCT116 and DLD-1 cells. At low FdUrd concentrations, because the amount of folate in the medium was limited, TYMS may have been inhibited effectively by the formation of a ternary complex when TYMS expression was downregulated. However, when TYMS expression was...
high, the amount of folate in the medium may have been insufficient to create ternary complexes and inhibit TYMS effectively. We investigated the relationship between the concentration of LV and the efficacy of FdUrd. In cells treated with control siRNA, addition of LV enhanced the cytotoxicity of FdUrd, whereas downregulation of TYMS abolished this effect (Fig 3). As expected, when TYMS expression was high (i.e., with control siRNA), additional LV was needed to effectively inhibit TYMS, whereas following treatment with TYMS siRNA, additional LV was not necessary. Tsujimoto et al. reported that the growth inhibitory effect of tegafur-uracil plus LV was superior to that of tegafur-uracil alone in tumors possessing high TYMS activity [7]. Similarly, our data show that the level of TYMS expression is important to determine whether additional LV is necessary to enhance the cytotoxicity of FdUrd. O’Dwyer et al. reported that addition of LV increased the side effects of 5-FU in conjunction with enhanced
efficacy [8]. Thus, minimizing the dose of LV required to inhibit TYMS by forming a ternary complex is considered a desirable aspect of combined 5-FU and LV treatment. Because the available folates may be saturated in tumors with low TYMS expression, thereby limiting the formation of ternary complexes even at low doses of LV, controlling the amount of LV administered in relation to the tumor folate level may improve the balance between the safety and efficacy of 5-FU treatment.

FOLR1 imports folic acid into cells and increases the folate pool (Fig 1). Karasawa et al. reported that expression of FOLR1 was decreased in a 5-FU-resistant human colon cancer cell line compared to its parental cell line, DLD-1 [9]. Consistent with their results, we found that the anti-proliferative efficacy of FdUrd was decreased by downregulating FOLR1 (Fig 4). These
results suggest that decreasing the amount of FOLR1 reduces the folate pool and inhibits the formation of ternary complexes.

Kalmbach et al. reported a del/del polymorphism in DHFR that diminished the capacity of the enzyme to reduce folic acid and limited the assimilation of folic acid into cellular folate stores at both high and low folic acid intakes [10]. Consistent with their results, our data showed that downregulation of DHFR suppressed the efficacy of FdUrd (Fig 5). Our findings suggest that abnormal folate metabolism resulting from inefficient functioning of DHFR, which synthesizes FH4 by reducing FH2 (Fig 1), affects the folate pool and results in limited folic acid assimilation.

Some studies have reported associations between MTHFR polymorphisms that reduced enzyme activity and the clinical outcome in CRC patients [11–13], whereas others have not [14–16]. In our study, suppression of MTHFR expression reduced the efficacy of FdUrd in HCT116 cells (Fig 8). Because MTHFR converts 5,10-CH2-FH4 to 5-CH3-FH4, downregulation of this enzyme may increase the formation of the ternary complex (Fig 1). However, Kawakami et al. reported that 5,10-CH2-FH4 and FH4 levels were lower in tumors with MTHFR polymorphisms [17]. Similar to the DHFR del/del polymorphism, the inefficient functioning of MTHFR affects the folate pool, decreasing its ability to assimilate folic acid.

GART and MTHFD1 are involved in folic acid metabolism. However, to our knowledge, no previous study on GART and MTHFD1 has linked them to the 5-FU response. We found a relationship between the efficacy of FdUrd and the expression of both GART and MTHFD1. Similar to FOLR1, DHFR, and MTHFR, downregulation of GART or MTHFD1 suppressed the efficacy of FdUrd (Figs 6 and 7).

Except for TYMS, downregulation of the folic acid-metabolizing enzymes that we investigated decreased the efficacy of FdUrd. Because downregulation of folic acid metabolizing enzymes results in reduction and imbalance of the folate pool, these results were expected. However, our study was limited to in vitro experiments using CRC cell lines. To confirm the relationship between the efficacy of 5-FU and folate metabolism-related molecules, a clinical study is necessary. Importantly, based on our data, further investigation of all factors evaluated in this study is warranted.
In conclusion, the results of the current study indicated that clarifying the relationship between folate metabolism-related enzymes and the efficacy of 5-FU treatment is important for predicting the effect of a combination therapy involving 5-FU and LV. With an improved understanding of this relationship, better therapy for CRC using 5-FU can be designed.

Supporting Information

S1 Table. Efficiency of silencing of folic acid metabolism-related genes by siRNA. The expression of mRNA was evaluated at different time points after transfection with siRNA. Data are presented as percentages of fold changes of mRNA expression following application of gene-specific siRNA relative to control siRNA.

(DOCX)

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Supervision: TT.
Validation: HT KT.
Visualization: HT.
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