Effect of the Methylenetetrahydrofolate Reductase C677T Polymorphism on Chemosensitivity of Colon and Breast Cancer Cells to 5-Fluorouracil and Methotrexate

Kyoung-Jin Sohn, Ruth Croxford, Zoe Yates, Mark Lucock, Young-In Kim

Background: Although single nucleotide polymorphisms may be potentially important pharmacogenetic determinants of cancer therapy, functional evidence regarding their relevance is currently lacking. The C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene is associated with changes in cellular composition of folates. We hypothesized that this polymorphism may modulate the cytotoxic effect of 5-fluorouracil (5FU) and methotrexate (MTX), two commonly used chemotherapeutic agents for colon and breast cancers, because the modes of action of 5FU and MTX are critically dependent on cellular composition of folates. Methods: Human HCT116 colon and MDA-MB-435 breast cancer cells were stably transfected with wild-type or mutant 677T human MTHFR cDNA. MTHFR enzyme activity and thermolability, intracellular folate composition, growth rate, and catalytic thymidylate synthase activity were determined. In vitro chemosensitivity of 5FU to 5FU and MTX was determined using the sulforhodamine B assay. In vivo chemosensitivity of HCT116 cells to 5FU was determined in nude mice. Results: Compared with cells expressing the wild-type MTHFR, HCT116 and MDA-MB-435 cells expressing the mutant 677T MTHFR had decreased MTHFR activity, MTHFR thermolability, changed intracellular folate distribution, accelerated cellular growth rate, and increased thymidylate synthase activity. The MTHFR 677T mutation increased chemosensitivity of colon and breast cancers to 5FU, but decreased chemosensitivity of breast cancer cells to MTX. In nude mice, xenografts expressing the mutant 677T MTHFR grew faster, but were more sensitive to 5FU, than xenografts expressing the wild-type protein. Conclusions: Our data provide evidence that the MTHFR C677T polymorphism affects the concentration and intracellular distribution of folates and changes the growth and chemosensitivity of colon and breast cancer cells. The MTHFR C677T polymorphism may be a useful pharmacogenetic determinant for providing rational and effective tailored chemotherapy. [J Natl Cancer Inst 2004;96:134–44]

Intracellular folate homeostasis depends on 5,10-methylenetetrahydrofolate reductase (MTHFR), a critical enzyme in folate metabolism that catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF) (Fig. 1) (1,2). The substrate 5,10-methyleneTHF is required for DNA synthesis and for maintaining the balance of the nucleotide pool, whereas 5-methylTHF is required for methylation reactions, including the methylation of homocysteine to methionine and the maintenance of DNA methylation patterns (Fig. 1) (1,2).

A mutation (C→T at nucleotide position 677) in the MTHFR gene, which results in an alanine to valine substitution, has been identified (1,2). This mutation reduces MTHFR activity and increases thermolability of MTHFR, which leads to lower levels of 5-methylTHF, an accumulation of 5,10-methyleneTHF, and increases in plasma homocysteine levels in individuals with a marginal folate status (2–7). The resulting changes in cellular composition of one-carbon folate derivatives may also lead to DNA hypomethylation (2–7). The MTHFR C677T polymorphism is a common mutation, with an allele frequency of about 35% in the general North American population (2,4,5). The polymorphism occurs frequently among Caucasian and Asian populations, with rates of ~12%–15% for individuals who are homozygous (TT) for the mutation and up to 50% for individuals who are heterozygous (CT) (8). The MTHFR C677T polymorphism may modulate the risk of cardiovascular disease, neural tube defects, and several cancers, although evidence to support these purported relationships has been equivocal (8).

An accumulation of 5,10-methyleneTHF resulting from the MTHFR C677T polymorphism (6) may also have an effect on the response of cancer cells to two commonly used chemotherapeutic agents, 5-fluorouracil (5FU) and methotrexate (MTX), because the activity of both drugs is dependent on a competitive interaction with folate metabolism. 5,10-MethyleneTHF is the methyl donor for the nonreversible methylation, catalyzed by thymidylate synthase, of deoxyuridine-5'-monophosphate (dUMP) to deoxythymidine-5'-monophosphate (dTMP), a precursor for DNA synthesis (Fig. 1). 5,10-MethyleneTHF is also involved in de novo purine biosynthesis (Fig. 1).

Although, in general terms, 5FU is considered to be a folate antimetabolite, it has several potential cytotoxic mechanisms. Two metabolites of 5FU, 5-fluoro-2'-deoxyuridine-5'-triphosphate and 5-fluorouridine-5'-triphosphate, can be incorporated into DNA and RNA, respectively, resulting in DNA instability and interfering with RNA processing and function (9). 5FU can also form a ternary complex involving 5-fluoro-2'-deoxyuridine-5'-monophosphate (5FdUMP; the active metabolite of 5FU), thymidylate synthase, and 5,10-methyleneTHF. The formation of this complex thereby inhibits thymidylate synthase activity, which subsequently depletes intracellular thymidylate levels and...
ultimately suppresses DNA synthesis (Fig. 1) (9). Leucovorin (also known as folic acid) or 5'-formylTHF, a precursor of 5,10-methyleneTHF, potentiates the cytotoxic effect of 5FU by stabilizing the inhibitory 5,10-methyleneTHF–thymidylate synthase–5FdUMP ternary complex (9). Therefore, the MTHFR C677T polymorphism, which increases intracellular concentrations of 5,10-methyleneTHF, may increase the cytotoxic effect of 5FU by increasing the formation and stability of the 5,10-methyleneTHF–thymidylate synthase–5FdUMP ternary complex.

By contrast, the cytotoxic effect of MTX may be compromised by an accumulation of intracellular 5,10-methyleneTHF resulting from the MTHFR C677T polymorphism. MTX inhibits dihydrofolate reductase, decreases intracellular 5,10-methyleneTHF levels for thymidylate biosynthesis, and directly inhibits purine biosynthesis. The accumulated dihydrofolate can inhibit both thymidylate synthase and enzymes involved in purine biosynthesis. Increased intracellular concentrations of 5,10-methyleneTHF counteract the mechanism of action of MTX.

MTHFR C677T polymorphism may be an important pharmacogenetic determinant of predicting response to 5FU and MTX. Identification of such a pharmacogenetic determinant would thereby enable physicians to provide rational and effective tailored chemotherapy to patients with colon and breast cancers. To test our hypothesis, we generated an in vitro model of the MTHFR C677T polymorphism in colon and breast adenocarcinoma cells and determined their chemosensitivity to 5FU and MTX.

METHODS

Cell Lines and Culture

Human colon adenocarcinoma HCT116 cells were purchased from the American Type Culture Collection (Manassas, VA), and human breast adenocarcinoma MDA-MB-435 cells were provided by Dr. M. Archer (University of Toronto, Toronto, Canada). Both cell lines were grown in RPMI-1640 medium (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin at 100 U/mL, and streptomycin at 100 μg/mL (complete medium). Cultures were maintained at 37°C in 5% CO2.

MTHFR Genotyping and Construction and Transfection of MTHFR Expression Vectors

The analysis of the MTHFR C677T mutation in HCT116 and MDA-MB-435 cells was performed by real-time polymerase chain reaction (PCR) as described (11). We constructed three expression vectors for use in this study. The first vector expressed wild-type MTHFR, the second vector expressed a mutant 677T MTHFR, and the third vector expressed an antisense MTHFR that functioned as a transdominant negative control. The full-length human MTHFR (hMTHFR) cDNA (2.0 kilobases [kb]) was cloned from human colon adenocarcinoma Caco-2 cell total RNA by reverse transcription using the MTHFR-specific primer 5'-GGAGTGCTTACCACACGG-3' and followed by PCR amplification using the above primer as the antisense primer and 5'-AACCCACGGCATGTTGAA-3' as the sense primer as described previously (12). The PCR product was first subcloned into the pBluescript SK(+) vector (Stratagene, Cambridge, U.K.). Four independent PCR clones were randomly selected and sequenced completely to avoid selecting clones with PCR-introduced sequence errors.

A thymine mutation at position 677 was introduced in the hMTHFR cDNA using PCR-based, site-directed mutagenesis (13). This method uses four primers—two gene-specific (sense: 5'-AACCCACGGCATGTTGAA-3', antisense: 5'-CCTGGAT GGGAAGATCC-3') and two mutated (sense: 5'-AAGGTT GTCTCGGAGTGATCATCATCATGACGAG-3', antisense: 5'-GAGTGGAAATGCAATCCTCCGAGACACCTTCC-3'). Five nanograms of the hMTHFR cDNA was used as template, and 7.5 μM gene-specific primer and 0.75 μM mutated primer, respectively, were used. The wild-type and mutant 677T hMTHFR cDNAs were subcloned into the eukaryotic expression vector pIRESneo (Clontech, Palo Alto, CA) containing a CMV promoter and a neomycin resistance gene expression cassette. To generate the antisense vector that functioned as a positive control for impaired MTHFR expression, the full-length hMTHFR cDNA was subcloned into the pIRESneo vector in the antisense orientation. The correct integration, orientation, and sequences of the wild-type,
mutant 677T, and antisense hMTHFR cDNAs were confirmed by predicted fragment sizes after multiple restriction enzyme digestions and DNA sequencing.

The pIRESe vectors containing the wild-type, mutant 677T, or antisense hMTHFR cDNA was stably transfected into HCT116 and MDA-MB-435 cells using Lipofectin (Invitrogen) according to the manufacturer’s recommended protocol. In a separate transfection, HCT116 and MDA-MB-435 cells were stably transfected with empty pIRESe vector (vector alone; endogenous hMTHFR). Transfected cells were incubated in the presence of neomycin (500 μg/mL; Invitrogen) to select for cells that expressed the various plasmids. After a population of cells was selected, individual clonal cell lines were isolated and expanded. Cells were maintained in complete medium supplemented with neomycin at 500 μg/mL. Several clones expressing the wild-type, 677T mutant, antisense hMTHFR cDNA, and empty vector from each cell line were selected at random for further analysis. Comparisons were made between cells expressing wild-type and 677T mutant hMTHFR and between cells expressing antisense and endogenous hMTHFR.

**Western Blot Analysis**

Total cellular lysates were obtained by incubating cells in RIPA solution containing protease inhibitors (phenylmethylsulfonyl fluoride at 0.1 mg/mL, aprotinin at 2 mg/mL) (Roche Diagnostics, Laval, Quebec, Canada). Supernatants were collected after centrifugation at 18,000g for 30 minutes at 4°C. Protein concentrations were determined using a protein assay kit (Bio-Rad, Mississauga, Ontario, Canada). Fifty micrograms of total cellular protein from each cell line was separated on a 12% sodium dodecyl sulfate–polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with phosphate-buffered saline (PBS) containing 5% skim milk for 2 hours at room temperature. To detect MTHFR protein expression, the membranes were incubated with a rabbit polyclonal antibody against amino acids 372–395 of hMTHFR (Zymed, San Francisco, CA) at a dilution of 1:2000 in PBS containing 5% skim milk and 0.1% Tween 20 for 16 hours at 4°C. To detect thymidylate synthase protein expression, the membranes were incubated with a sheep polyclonal antibody against human thymidylate synthase (Rockland Immunodiagnostic, Gilbertsville, PA) at a dilution of 1:300 in PBS containing 5% skim milk and 0.1% Tween 20 for 16 hours at 4°C. The MTHFR and thymidylate synthase proteins were visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ). To confirm that the proteins were loaded equally, the membranes were stripped and reprobed with a human anti-β-actin antibody (Sigma Aldrich Canada, Oakville, Ontario, Canada) at a dilution of 1:3000.

**MTHFR Enzyme Assay**

The 14C-labeled methyltetrahydrofolate–menadione oxidoreductase assay was used to measure specific MTHFR activity as described (14). Each assay used 100 μg of protein extract per cell line. The extracts were prepared as described (15). To determine MTHFR thermolability, the protein extracts were incubated at 46°C for 5 minutes and residual MTHFR enzyme activity was measured as described (15).

**Intracellular Folate Concentrations and Distributions**

Cell pellets containing 20 × 10⁶ cells were protected against labile folate oxidation by the addition of 100 μL of freshly made ascorbate (20 g/L). The cell suspension was then vortex mixed and stored in 100-μL aliquots at −80°C until analyzed. Immediately before intracellular folate levels were measured, an aliquot of this cell–ascorbate preparation was thawed, and 50 μL of ascorbate (20 g/L) was added rapidly to the preparation and vortex mixed for 20 seconds. To this preparation, a total of 12.5 μL of 11 M HClO₄ was added, and the sample was mixed for 20 seconds. The preparation was then neutralized with the same volume of equimolar KOH, mixed, and spun at 13 000g for 2 minutes. The supernatant was collected, and 100 μL was injected into a liquid chromatograph for spectral confirmation of folate coenzyme identified using photodiode array detection based on the method of Lucock et al. (16). An isocratic high-performance liquid chromatography system with fluorescence detection (λex = 310 nm, λem = 352 nm) (17) was used to quantify individual intracellular folate coenzyme species from 17.5 μL of supernatant. Intra-batch coefficient of variation (CV) values for the extraction and measurement of endogenous methyl-THF and formylTHF polyglutamates were as follows: for methylTHF, CV = 4.70, 3.84, 2.94, 7.85, and 3.53 for polyglutamate chain lengths of 1–5 glutamate moieties, respectively; for formylTHF, CV = 18.82, 4.28, 4.13, 3.85, and 4.84 for polyglutamate chain lengths of 1–5 glutamate moieties, respectively.

**Doubling-Time Calculation**

Cells (8000 per well) were plated in 96-well plates and grown in RPMI-1640 medium with 10% fetal bovine serum for 72 hours. The cell population was determined using the sulforhodamine B (SRB) optical density (OD) measurement assay (18,19). The growth rate constant k was derived using an equation N/N₀ = e^kt, where N₀ is the optical density of cells at time zero and N is the optical density of cells at 72 hours. The same equation was used to calculate the doubling time t by setting N/N₀ = 2. All analyses were performed in triplicate, and three replicate experiments were performed.

**In Vitro Chemosensitivity**

In vitro chemosensitivity of cells stably transfected with the different MTHFR constructs was determined using a modified SRB protein assay as described (18,19). Briefly, 8000 cells per 100 μL medium per well were seeded in triplicate in 96-well flat-bottom plates. After 24 hours, an additional 100 μL of medium containing MTX (Schircks, Jona, Switzerland), 5FU alone, or 5FU (InvivoGen, San Diego, CA) in combination with leucovorin (Sigma Aldrich) was added, and the cells were cultured for an additional 72 hours. The concentration of MTX was varied, with concentrations ranging from 3.5 × 10⁻⁶ M to 5 × 10⁻⁶ M. The concentration of 5FU was varied, with concentrations ranging from 1.5 × 10⁻⁶ M to 25 × 10⁻⁶ M, whereas the concentration of leucovorin was held constant at 5 × 10⁻⁶ M. Leucovorin was added to simulate the standard 5FU-based chemotherapy used in the treatment of colorectal and breast cancers (9). After 72 hours, cells were fixed with trichloroacetic acid and stained with SRB protein dye. The dye was solubilized, and the optical density of the solution measured at 595 nm. The results were expressed as a percentage of cell survival on the basis of
the difference between the OD at the start and end of drug exposure, according to the formula (20):

\[
\text{Survival} = \frac{[\text{OD}_{\text{drug}}/\text{OD}_{\text{start drug exposure}}] - 1}{[\text{OD}_{\text{no drug}}/\text{OD}_{\text{start drug exposure}}] - 1} \times 100\%.
\]

IC\textsubscript{50} values (i.e., the drug concentration that corresponded to a reduction in cell survival by 50\% compared with the survival of untreated control cells) were calculated from plots of drug concentration versus proportion of cells that survived.

**In Vivo Chemosensitivity**

Forty 6-week-old female BALB/c nu/nu mice (Charles River, Wilmington, MA) were randomly assigned to one of four groups (group 1, mice injected with xenografts expressing the wild-type MTHFR and treated with 0.9\% NaCl; group 2, mice injected with xenografts expressing the mutant MTHFR and treated with 0.9\% NaCl; group 3, mice injected with xenografts expressing the wild-type MTHFR and treated with 5FU; and group 4, mice injected with xenografts expressing the mutant MTHFR and treated with 5FU). Each group (n = 10) received intraperitoneal injections of 0.9\% NaCl; group 3, mice injected with xenografts expressing the mutant MTHFR and treated with 0.9\% NaCl; group 2, mice injected with xenografts expressing the mutant MTHFR and treated with 5FU; and group 4, mice injected with xenografts expressing the mutant MTHFR and treated with 5FU). Each group (n = 10) received a subcutaneous injection of the flank of HCT116 cells expressing wild-type or mutant 677T MTHFR (5 \times 10^6 cells per mouse) in 0.2 mL serum-free RPMI-1640 medium. The cells were washed twice by centrifugation (1000 \times g for 5 minutes) in serum-free RPMI-1640 medium before injection. When xenografts reached a volume of 80–200 mm\(^3\) (approximately 3 weeks after injection), 5FU (40 mg/kg/day) dissolved in 0.2 mL of 0.9\% NaCl was administered by intraperitoneal injection for 5 consecutive days a week for 2 weeks, as described (21). Leucovorin (4 mg/kg/day) was administered 1 hour before 5FU administration by intraperitoneal injection, as described (21). Control mice for each group received intraperitoneal injections of 0.9\% NaCl. The tumors were measured with a caliper twice a week. The estimated tumor volume (V) was calculated by the following formula (22):

\[
V = \frac{W^2 \times L}{2} \times 0.5,
\]

where W represents the largest tumor diameter in centimeters and L represents the next largest tumor diameter. The individual relative tumor volume (RTV) was calculated as follows:

\[
\text{RTV} = \frac{V_t}{V_0},
\]

where V\(_t\) is the volume in cubic millimeters at a given time and V\(_0\) is the volume at the start of treatment. Results are expressed as the mean daily percent change in tumor volume for each group of mice.

**Thymidylate Synthase Catalytic Enzyme Activity**

The catalytic activity of thymidylate synthase was determined by \(^{3}\)H release that occurred during the conversion of [\(^{3}\)H]-dUMP to dTMP, as described (23). Briefly, 10 \mu M [\(^{3}\)H]-dUMP and 350 \mu M methyleneTHF (final concentration) were added to 150 \mu g of total cellular protein in a total volume of 50 \mu L of Tris–HCl buffer (pH 7.4) for 1 hour at 37 °C. The reaction was ended by the addition of 50 \mu L of 35\% trichloroacetic acid. After the addition of 250 \mu L of 10\% activated charcoal in 0.2 \text{ M HCl}, which bound the unreacted [\(^{3}\)H]-dUMP, the mixture was centrifuged for 30 minutes, the supernatant was collected, and the amount of radioactivity in the supernatant was measured.

**Statistical Analysis**

Comparisons among cells expressing mutant and wild-type MTHFR were determined using Student’s t test. For the in vitro chemosensitivity analyses, plots of percentage of survival versus dose demonstrated S-shaped curves, and therefore the logit transformation \([\text{logit}(p) = \ln(p/(1-p))]\) was used. Ordinary least-squares regression was used to model the effect of log(dose) of chemotherapy and cell type (wild-type versus mutant 677T MTHFR or antisense versus endogenous MTHFR) on the logit-transformed proportion of cells that survived at each dose. The interaction between cell type and log(dose) was included in the model to test the hypothesis that the cell types were differentially sensitive to chemotherapy. IC\textsubscript{50} doses and their 95\% confidence intervals (CIs) were calculated on the log-scale from the regression results, as described (24), and then back-transformed to the original scale for reporting. For the in vivo chemosensitivity analyses, because the tumor volumes were skewed, the data were log-transformed before analysis, producing a dependent outcome variable that was linear when plotted against time (measured in days). For each mouse, the rate of tumor growth was estimated using ordinary least-squares regression, with log(volume) as the dependent variable and day as the independent variable. The resulting slopes estimate the change in log(volume) per day and, when back-transformed, estimate the growth rate per day. The slope estimates were used as the dependent variable in a two-way analysis of variance. The two independent factors were tumor type (wild-type versus mutant 677T) and treatment (chemotherapy versus saline). An interaction effect was included in the analysis to test the hypothesis that the effect of chemotherapy differed for the two cell types. For all analyses, results were considered statistically significant if two-tailed P values were less than .05. Analyses were performed using SAS, version 8 (SAS Institute, Cary, NC).

**RESULTS**

**Effect of the MTHFR 677T Mutation on MTHFR Activity, MTHFR Thermolability, and Cellular Composition of Folates**

Parental HCT116 and MDA-MB-435 cell lines were both heterozygous (CT) for the MTHFR 677T genotype. Compared with untransfected parental cells, specific MTHFR enzyme activity and total intracellular folate concentrations from mock-transfected HCT116 and MDA-MB-435 cells were similar.

The wild-type and mutant 677T MTHFR proteins were abundantly expressed in HCT116 and MDA-MB-435 cells transfected with the wild-type and mutant 677T MTHFR cDNAs (Fig. 2, A). Specific MTHFR enzyme activity was approximately 35\% lower (95\% CI = 31\% to 39\%) in colon and breast cancer cells expressing the mutant 677T MTHFR than in cells expressing the wild-type MTHFR protein (P < .001). In both HCT116 and MDA-MB-435 cells, the expression of the mutant 677T MTHFR was associated with statistically significantly higher thermolability than expression of the wild-type MTHFR, because there was statistically significantly lower residual MTHFR activity after heating cell lysates at 46 °C (Fig. 2, B and C; P < .001). As a positive control, HCT116 cells stably transfected with the vector containing the full-length hMTHFR cDNA in the antisense orientation were compared with HCT116 cells transfected with the vector without an insert. Both western analysis and the specific MTHFR enzyme assay confirmed that, compared with cells transfected with the vector alone, there was a statistically significant inhibition of MTHFR protein expression and activity in HCT116 cells transfected with the antisense vector (Fig. 2, A and D; P < .001).
Consistent with the biochemical consequence of impaired MTHFR (Fig. 1), the relative proportion of methylTHF to the total folate pool was lower by 12.7% and 10.6% in HCT116 and MDA-MB-435 cells expressing the mutant 677T MTHFR, respectively, than in corresponding cells expressing the wild-type MTHFR (Table 1). Furthermore, the relative proportions of formylTHF and methenylTHF (and thus, the proportion of methyleneTHF; Fig. 1) were higher by 12.7% and 10.6% in HCT116 and MDA-MB-435 cells expressing the mutant 677T MTHFR, respectively, than in corresponding cells expressing the wild-type MTHFR (Table 1). The proportion of THF was similar between cells expressing the mutant 677T MTHFR and those expressing wild-type MTHFR (Table 1). HCT116 cells expressing the antisense MTHFR had a higher proportion of formylTHF and methenylTHF and a lower portion of methylTHF than those expressing endogenous MTHFR (Table 1), which was consistent with the changes in cellular composition of folate derivatives associated with expression of the mutant 677T MTHFR. Although the total folate concentration in HCT116 cells expressing the antisense MTHFR was 26% lower than that in cells expressing endogenous MTHFR, there was no appreciable difference between HCT116 cells expressing the mutant 677T and those expressing wild-type MTHFR (Table 1). Total intracellular folate concentration in MDA-MB-435 cells expressing the mutant 677T MTHFR was 18% lower than that in cells expressing the wild-type MTHFR (Table 1). These findings tend to support what might be predicted on the basis of biochemical theory and the limited evidence regarding the functional consequences of 677T MTHFR polymorphism.

Effect of the MTHFR 677T Mutation on Growth Rate

Both HCT116 and MDA-MB-435 cells expressing the mutant 677T MTHFR grew faster than corresponding cells expressing the wild-type MTHFR. The doubling time of HCT116 and MDA-MB-435 cells expressing the mutant 677T MTHFR was statistically significantly shorter than that for corresponding cells expressing the wild-type MTHFR (HCT116 cells, 35.4 ± 0.1

Table 1. Intracellular folate concentration and composition in HCT116 colon and MDA-MB-435 breast cancer cells expressing endogenous, antisense, mutant 677T, and wild-type 5,10-methylenetetrahydrofolate reductase (MTHFR)*

<table>
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<tr>
<th>Cell line</th>
<th>Clone</th>
<th>Total folate ng/20 × 10⁶ cells†</th>
<th>% of total folate</th>
<th>MethylTHF</th>
<th>FormylTHF/methenylTHF</th>
<th>THF</th>
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*THF = tetrahydrofolate; VA = vector alone (endogenous); WT = wild-type; 677T = C→T mutation at nucleotide 677.
†Native intracellular folate enzymes were separated by isocratic high-performance liquid chromatography. Photodiode array detection was used for spectral characterization of the individual forms of the folates between 200 and 400 nm. Quantitation of folates was facilitated by fluorescence detection using λmax = 310 nm, λexc = 352 nm, as described (16). Individual pools of each one-carbon form of folate are expressed as a percentage of the total intracellular folate pool. Values given are based on a single extraction for each cell line.
hours versus 38.2 ± 1.1 hours, \( P < .001 \); MDA-MB-435 cells, 45.0 ± 0.7 hours versus 52.4 ± 2.0 hours, \( P < .001 \). By contrast, growth of HCT116 cells expressing the antisense MTHFR was inhibited, and the doubling time statistically significantly increased compared with that for the corresponding cells expressing endogenous MTHFR (30.2 ± 0.8 hours versus 25.4 ± 0.2 hours; \( P < .001 \)). The accelerated growth rate associated with the MTHFR 677T mutation is consistent with the known biochemical ramifications of the MTHFR C677T polymorphism, which results in an accumulation of 5,10-methyleneTHF (6,7) and a consequent increase in thymidylate and purine biosynthesis. However, the inhibitory effect of antisense MTHFR on growth rate is not readily explained by the changes in intracellular folate composition associated with MTHFR inhibition.

**Effect of the MTHFR 677T Mutation on Chemosensitivity of HCT116 and MDA-MB-435 Cells to 5FU In Vitro and In Vivo**

We hypothesized that the MTHFR C677T polymorphism, which increases intracellular concentrations of 5,10-methyleneTHF, would enhance the cytotoxic effect of 5FU by increasing the formation and stability of the 5,10-methyleneTHF–thymidylate synthase–FdUMP ternary complex. We first tested this hypothesis in an *in vitro* chemosensitivity assay. The chemosensitivity of HCT116 and MDA-MB-435 cells expressing the mutant 677T MTHFR to 5FU plus leucovorin was statistically significantly increased compared with the corresponding cells expressing the wild-type MTHFR at each concentration of 5FU tested (\( P < .001 \) for both; Fig. 3, A and B). The IC\(_{50}\) value for 5FU was statistically significantly lower in HCT116 and MDA-MB-435 cells expressing the mutant 677T MTHFR than in the corresponding cells expressing the wild-type MTHFR (4.2 \( \mu M \) [95% CI = 4.0 to 4.4 \( \mu M \)] versus 9.3 \( \mu M \) [95% CI = 8.8 to 9.8 \( \mu M \)] for HCT116 cells and 7.2 \( \mu M \) [95% CI = 6.6 to 7.8 \( \mu M \)] versus 31.6 \( \mu M \) [95% CI = 26.1 to 40.0 \( \mu M \)] for MDA-MB-435 cells; \( P < .001 \)). In *in vitro* chemosensitivity to 5FU plus leucovorin was statistically significantly increased in HCT116 cells expressing the antisense MTHFR compared with cells expressing endogenous MTHFR (\( P = .003 \); Fig. 3, C). The corresponding IC\(_{50}\) value was statistically significantly lower in HCT116 cells expressing the antisense MTHFR than in cells expressing endogenous MTHFR (1.7 \( \mu M \) [95% CI = 1.6 to 2.1 \( \mu M \)] versus 12.3 \( \mu M \) [95% CI = 10.7 to 14.3 \( \mu M \)]; \( P < .001 \)).

Chemosensitivity to 5FU alone was also statistically significantly increased in HCT116 and MDA-MB-435 cells expressing the mutant 677T MTHFR compared with the corresponding cells expressing the wild-type MTHFR (\( P < .001 \); Fig. 3, D and E). The IC\(_{50}\) value for 5FU alone was statistically significantly lower in HCT116 and MDA-MB-435 cells expressing the mutant 677T MTHFR than in the corresponding cells expressing the wild-type MTHFR (4.7 \( \mu M \) [95% CI = 4.4 to 5.0 \( \mu M \)] versus 8.4 \( \mu M \) [95% CI = 8.0 to 9.0 \( \mu M \)] for HCT116 cells, and 6.4 \( \mu M \) [95% CI = 5.6 to 7.4 \( \mu M \)] versus 25.6 \( \mu M \) [95% CI = 22.3 to 29.4 \( \mu M \)] for MDA-MB-435 cells; \( P < .001 \). *In vitro* chemosensitivity to 5FU alone was also statistically significantly increased in HCT116 cells expressing the antisense MTHFR compared with cells expressing endogenous MTHFR (\( P < .001 \); Fig. 3, F). The corresponding IC\(_{50}\) value was statistically significantly lower in HCT116 cells expressing the antisense MTHFR than in cells expressing endogenous MTHFR (6.2 \( \mu M \) [95% CI = 5.7 to 6.8 \( \mu M \)] versus 20.3 \( \mu M \) [95% CI = 18.6 to 22.1 \( \mu M \)]; \( P < .001 \)).

We next tested whether the MTHFR 677T mutation affected the *in vivo* chemosensitivity of HCT116 cells to 5FU plus leucovorin in nude mice. Leucovorin was added to simulate the standard 5FU-based chemotherapy used in the treatment of colorectal cancer and, thus, *in vivo* chemosensitivity to 5FU alone was not tested. When the growth rates of the mutant 677T MTHFR and wild-type xenografts in mice injected with saline were compared, it was evident that the mutant 677T MTHFR xenografts grew faster than the wild-type xenografts (average growth rate = 17.6%/day [95% CI = 12.7 to 22.7%/day] versus 9.3%/day [95% CI = 6.1 to 12.6%/day]; \( P = .007 \); Fig. 4, A). The growth rate of the HCT116 xenografts expressing the mutant 677T MTHFR was inhibited more effectively by 5FU plus leucovorin (78% inhibition) than was the growth of those expressing the wild-type MTHFR (36% inhibition) (\( P = .008 \); Fig. 4, B). The *in vivo* chemosensitivity of these results support the *in vitro* observations that cancer cells expressing the mutant 677T MTHFR have faster growth rates than cells expressing wild-type MTHFR, but that cells expressing the mutant 677T MTHFR have increased sensitivity to 5FU plus leucovorin.

**Effect of the MTHFR 677T Mutation on Chemosensitivity of HCT116 and MDA-MB-435 Cells to MTX In Vitro**

We next hypothesized that the cytotoxic effect of MTX would be compromised by an accumulation of intracellular 5,10-methyleneTHF resulting from the MTHFR C677T polymorphism, because this would counteract the mode of MTX action, which depletes intracellular methyleneTHF for thymidylate and purine biosynthesis. We tested this hypothesis by comparing the *in vitro* chemosensitivity of HCT116 and MDA-MB-435 cells to MTX. *In vitro* chemosensitivity of MDA-MB-425 cells expressing the mutant 677T MTHFR to MTX was statistically significantly decreased compared with the chemosensitivity of cells expressing wild-type MTHFR (\( P = .011 \); Fig. 3, H). The IC\(_{50}\) value for MTX was statistically significantly higher in MDA-MB-435 cells expressing the mutant 677T MTHFR than in cells expressing the wild-type MTHFR (27.2 nM [95% CI = 22.5 to 34.5 nM] versus 8.6 nM [95% CI = 7.0 to 10.2 nM]; \( P < .001 \)). By contrast, there was no statistically significant difference in chemosensitivity to MTX between HCT116 cells expressing the mutant 677T MTHFR and those expressing wild-type MTHFR (\( P = .98 \); Fig. 3, G). The IC\(_{50}\) value of MTX was not statistically significantly different between HCT116 cells expressing the mutant 677T MTHFR and those expressing wild-type MTHFR (3.7 nM [95% CI = 3.2 to 4.3 nM] versus 3.1 nM [95% CI = 2.6 to 4.0 nM]). However, *in vitro* chemosensitivity to MTX was statistically significantly decreased (\( P < .001 \); Fig. 3, I), and the IC\(_{50}\) value for MTX was statistically significantly increased (7.5 nM [95% CI = 7.0 to 8.1 nM] versus 6.2 nM [95% CI = 6.0 to 6.3 nM]; \( P < .001 \)) for HCT116 cells expressing the antisense MTHFR compared with cells expressing the endogenous MTHFR. Consistent with the predictable changes in intracellular composition of folates, the MTHFR C677T polymorphism decreased chemosensitivity of breast cancer cells to MTX, whereas this effect was observed only with the antisense MTHFR, and not with the C677T mutation, in colon cancer cells.
Effect of the MTHFR 677T Mutation on Thymidylate Synthase Catalytic Activity in HCT116 and MDA-MB-435 Cells

We hypothesized that increased intracellular levels of 5,10-methyleneTHF resulting from the MTHFR 677T mutation would increase thymidylate synthase catalytic activity by providing abundant amounts of the methyl donor for the methylation, catalyzed by thymidylate synthase, of dUMP to dTMP (Fig. 1). Thymidylate synthase protein expression was similar between HCT116 and MDA-MB-435 cells expressing the wild-type MTHFR and cells expressing the mutant 677T MTHFR or between HCT116 cells expressing the antisense MTHFR (AS) or endogenous MTHFR (VA) was measured using a modified sulfoboradamine B (SRB) protein assay as described (18,19). Cells (8000 cells per 100 μL of medium per well) were seeded in triplicate in 96-well flat-bottom plates. After 24 hours, an additional 100 μL of medium containing MTX, 5FU alone, or 5FU in combination with leucovorin was added, and the cells were cultured for an additional 72 hours. After 72 hours, cells were fixed with trichloroacetic acid and stained with the SRB protein dye. The dye was solubilized, and the optical density (OD) of the solution was measured at 595 nm. The results were expressed as a percentage of cell survival on the basis of the difference between the OD at the start and the OD at the end of drug exposure (20). Each point represents the mean of six replicates.

DISCUSSION

Single nucleotide polymorphisms (SNPs) have been used as markers of cancer risk, and several have been used to elucidate new biologic pathways of cancer development. More recently, several SNPs have emerged as important prognostic indicators for cancer patients and as promising pharmacogenetic determinants of response to cancer chemotherapy (25). The role of pharmacogenetics in cancer chemotherapy is important because it can guide the selection of optimal therapy for maximal efficacy and minimal toxicity (25). However, currently available clinical studies (25) do not provide conclusive evidence that SNPs have functional consequences, and in the absence of such data, the pharmacogenetic relevance of the SNPs remains generally uncertain. In this regard, the MTHFR C677T polymorphism is an ideal candidate for investigating the role of SNPs in the pharmacogenetics of cancer chemotherapy because of its biochemical effect on intracellular folate composition. Folate is critical for DNA synthesis, DNA repair, and epigenetic regulation. The present study has elucidated, for the first time, the functional pharmacogenetic consequences of the MTHFR C677T polymorphism with respect to response to chemotherapy in vitro and in vivo, providing proof of principle.

We hypothesized that increased intracellular concentrations of 5,10-methyleneTHF resulting from the MTHFR C677T polymorphism would increase the chemosensitivity of colon and
breast cancer cells to 5FU by increasing the formation and stability of the 5,10-methyleneTHF–thymidylate synthase–FdUMP ternary complex, with a consequent inhibition of thymidylate synthase and DNA synthesis. Furthermore, we hypothesized that cytotoxicity of MTX would be compromised by the MTHFR C677T polymorphism because of the increased intracellular concentrations of 5,10-methyleneTHF that ensure the flow of one-carbon units into thymidylate and purine biosynthesis. We developed an in vitro model of the MTHFR 677T mutation in colon and breast cancer cells with predictable functional consequences including decreased MTHFR activity, increased MTHFR thermolability, changes in the intracellular pool of folate derivatives (decreased methylTHF and increased formylTHF/methenylTHF), and increased thymidylate synthase activity. With this model, we showed that the MTHFR 677T mutation increased chemosensitivity of colon and breast cancer cells to 5FU and decreased chemosensitivity of breast cancer cells to MTX. For colon cancer cells, increased chemosensitivity to 5FU associated with the MTHFR 677T mutation was observed both in vitro and in vivo. There was a suggestion that the addition of leucovorin increased the chemosensitivity of colon and breast cancer cells expressing mutant 677T MTHFR to 5FU. The lack of effect of the MTHFR 677T mutation on chemosensitivity of colon cancer cells to MTX is not entirely surprising because colon cancer cells are generally not sensitive to MTX (26). Interestingly, however, antisense inhibition of MTHFR statistically significantly decreased chemosensitivity of colon cancer cells to MTX, raising the possibility that greater MTHFR inhibition than that achieved in our study may modulate chemosensitivity of colon cancer cells to MTX. Collectively, on the basis of the metabolic consequences of the changes in intracellular composition of folate derivatives, our study provides evidence that the MTHFR C677T polymorphism differentially modulates chemosensitivity of colon and breast cancer cells to chemotherapeutic agents, depending on their modes of action. Our data also suggest that antisense inhibition of MTHFR may be a potential target for increasing sensitivity of colon and breast cancer cells to 5FU-based chemotherapy. Thymidylate synthase is a critical target for 5FU and MTX, and its expression level is an important prognostic factor for colorectal and breast cancers and a predictor of chemosensitivity of cancer cells to 5FU and MTX. Tumor thymidylate synthase expression appears to be inversely related to prognosis in patients with colorectal and breast cancers (25). Careful analyses of the literature and emerging new evidence, however, suggest that high thymidylate synthase expression is a poor prognostic factor for untreated patients with colorectal cancer but a good prognostic factor for patients treated with adjuvant 5FU-based chemotherapy (27–30). By contrast, low thymidylate synthase expression seems to be a poor prognostic factor for patients treated with adjuvant 5FU-based chemotherapy (27–30). A large series of in vitro preclinical data collectively suggest a positive association between thymidylate synthase activity and 5FU sensitivity in colorectal and breast cancer cell lines (31–33). These in vivo (27–30) and in vitro (31–33) observations suggest that the most effective inhibition of thymidylate synthase will occur in cancer cells that are rapidly dividing and thus have high thymidylate synthase expression or activity. Our study indicates that the MTHFR 677T mutation and antisense MTHFR inhibition increase thymidylate synthase catalytic activity as a result of an increased supply of 5,10-methyleneTHF, the methyl donor for the methylation of dUMP to dTMP (Fig. 1). This mechanism is supported by the observation that thymidylate synthase protein expression was not statistically significantly different between colon and breast cancer cells expressing wild-type MTHFR and cells expressing mutant 677T MTHFR and between colon cancer cells expressing antisense MTHFR and cells expressing endogenous MTHFR. In cells treated with MTX, the MTHFR 677T mutation decreased chemosensitivity of breast cancer cells because the increased thymidylate synthase catalytic activity and intracellular 5,10-methyleneTHF concentrations enhanced thymidylate and purine biosynthesis, thereby countering the mode of MTX action. In cells treated with 5FU, the MTHFR 677T mutation increased chemosensitivity of colon and breast cancer cells because the increased 5,10-methyleneTHF concentrations and thymidylate synthase catalytic activity en-
hanced the formation and stability of the inhibitory 5,10-
methylenetetrahydrofolate–thymidylate synthase–FdUMP ternary complex,
thereby augmenting the mode of 5FU action.

Because the MTHFR C677T polymorphism occurs with an
allelic frequency of about 35%, its role in cancer pharmacoge-
netics has important health implications. For this polymorphism
to be used as a pharmacogenetic determinant in predicting re-
ponse to and toxicities from chemotherapy, the observed effects
of the MTHFR C677T polymorphism on sensitivity of colon
and breast cancers to 5FU and MTX need to be confirmed in human
studies. Recent human studies have suggested that the MTHFR
TT genotype may increase toxicity to MTX alone or in combi-
nation with other chemotherapeutic agents in patients undergoing
bone marrow transplantation (34), with leukemia (35), with
ovarian cancer (36), and with breast cancer (37). Another study
(38) involving 43 patients with metastatic colorectal cancer who
received 5FU and other fluoropyrimidine-based chemotherapy
has shown a statistically significant difference in the frequency of the
T MTHFR allele among responders versus nonresponders
\( P = 0.035 \), with an odds ratio of 2.86 (95% CI 1.06 to 7.73)
for a response in individuals with a T allele. However, the
differences in the proportion of objective responses among in-
dividuals with CC, CT, and TT genotypes did not reach statistical
significance, likely because of the small sample size. Large
clinical trials are therefore necessary to confirm the effect of the
MTHFR C677T polymorphism on treatment response and survival
in cancer patients receiving chemotherapy.

Although colon cancer cells expressing mutant 677T MTHFR
and cells expressing antisense MTHFR had similar biochemical
consequences, only cells expressing mutant 677T MTHFR had
an accelerated growth rate. This discrepancy in growth rate
between cells expressing 677T MTHFR and antisense MTHFR
is unclear. One possible explanation is related to total intracel-
ular folate concentrations. Colon cancer cells expressing anti-
sense and endogenous MTHFR had statistically significantly
lower total intracellular folate concentrations than cells express-
ing wild-type and mutant 677T MTHFR. Relative to changes in
the appropriate control cell populations, folate depletion in colon
cancer cells expressing antisense MTHFR was greater than that
in cells expressing mutant 677T MTHFR. Therefore, it is possi-
ble that the relative depletion of total intracellular folates might
explain the observed growth inhibition associated with the an-
tisense MTHFR, overriding the effects of an increased relative
proportion of 5,10-methylenetetrahydrofolate and increased thymidylate
synthase activity.

The effect of the MTHFR C677T polymorphism on total
tissue folate concentrations and intracellular folate composition,
particularly in the target organs such as the colon and breast, has
not been reported. Generally, the MTHFR C677T polymorphism
has been associated with lower plasma folate concentrations
than the wild-type genotype (2–5). However, data are equivocal
for the effect of the MTHFR C677T polymorphism on folate
concentrations in red blood cells (3,6,7,39–41), although the
MTHFR C677T polymorphism does appear to decrease meth-
rylTHF and increase formylTHF in red blood cells (6,42). In a
recent study (43), no statistically significant differences in total
folate content were observed in liver and brain tissues among
wild-type (Mthfr\(^{+/+}\)) , heterozygous (Mthfr\(^{+-}\)), and knockout
(Mthfr\(^{-/-}\)) mice, although the proportion of methylTHF in liver
and brain tissues was statistically significantly lower in
knockout mice than in wild-type mice. Our data show that,
compared with the wild-type MTHFR, the MTHFR C677T
polymorphism is associated with a decreased relative propor-
tion of methylTHF and an increased relative proportion of
formylTHF/methenylTHF (and thus a higher proportion of
methyleneTHF) in human colon and breast cancer cells. How-
ever, our data demonstrate that, compared with the wild-type
MTHFR, the MTHFR C677T polymorphism is associated
with lower total folate concentrations in breast, but not colon,
cancer cells. Collectively, our data and data from another
study (43) suggest that the effect of the MTHFR C677T
polymorphism on total tissue folate concentrations may be
highly variable and tissue-specific.

One potential limitation of our study is that the functional
effects of the MTHFR 677T mutation were determined in cells
expressing endogenous MTHFR (i.e., HCT116 and MDA-MB-
435 cells were heterozygous [CT] for MTHFR). However, comparisons were made between cells expressing the mutant 677T and wild-type MTHFR—both of which were statistically significantly overexpressed—likely overshadowing any effect of endogenous MTHFR. Furthermore, the functional effects of the MTHFR 677T mutation observed in our in vitro system were confirmed in an in vivo system of antisense MTHFR inhibition and in an in vivo model.¹

In conclusion, we provide evidence that the MTHFR C677T polymorphism may be an important pharmacogenetic determinant of 5FU- and MTX-based cancer chemotherapy. Our data suggest that the MTHFR C677T polymorphism may be a useful pharmacogenetic determinant for providing rational and effective tailored cancer chemotherapy. Furthermore, our data suggest that antisense inhibition of MTHFR may be a potential target for increasing chemosensitivity of colon and breast cancer cells to 5FU-based chemotherapy. The pharmacogenetics of the MTHFR C677T polymorphism may also be applied to other disease processes in which MTX, 5FU, and newer analogs are used for treatment (e.g., MTX in rheumatoid arthritis and inflammatory bowel disease).

REFERENCES


NOTES

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