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Background: Polyglutamylation of intracellular folates and antifolates (for example, methotrexate (MTX)) to polyglutamates. Polyglutamylated folates and antifolates are retained in cells longer and are better substrates than their monoglutamate counterparts for enzymes involved in one carbon transfer. Polyglutamylation of intracellular 5,10-methylene tetrahydrofolate may also enhance the cytotoxicity of 5-fluorouracil (5-FU) by allowing more efficient formation and stabilisation of the inhibitory ternary complex involving thymidylate synthase and a 5-FU metabolite.

Aim: We investigated the effects of FPGS modulation on the chemosensitivity of colon cancer cells to 5-FU and MTX.

Methods: Human HCT116 colon cancer cells were stably transfected with the sense or antisense FPGS cDNA or blank (control). FPGS protein expression and enzyme activity, growth rate, intracellular folate content and composition, and in vitro chemosensitivity to 5-FU and MTX were determined.

Results: Compared with cells expressing endogenous FPGS, those overexpressing FPGS had significantly faster growth rates and higher concentrations of total folate and long chain folate polyglutamates while antisense FPGS inhibition produced opposite results. FPGS overexpression significantly enhanced, whereas FPGS inhibition decreased, chemosensitivity to 5-FU. No significant difference in chemosensitivity to MTX was observed.

Conclusions: These data provide functional evidence that FPGS overexpression and inhibition modulate chemosensitivity of colon cancer cells to 5-FU by altering intracellular folate polyglutamylation, providing proof of principle. Thus FPGS status may be an important predictor of chemosensitivity of colon cancer cells to 5-FU based chemotherapy, and FPGS gene transfer may increase the sensitivity of colon cancer cells to 5-FU-based chemotherapy.
FPGS appears to play an important role in the sensitivity of cancer cells to antifolates and 5-FU and thus FPGS modulation might be a potential therapeutic target for increasing sensitivity of cancer cells to these chemotherapeutic agents. However, definitive functional evidence supporting the effect of FPGS modulation on the chemosensitivity of colon cancer cells to MTX and 5-FU is currently lacking. Therefore, we generated an in vitro model of FPGS modulation in colon cancer cells and determined the effect of FPGS overexpression and inhibition on the chemosensitivity of colon cancer cells to MTX and 5-FU.

MATERIALS AND METHODS

Cell line and culture

Human colon adenocarcinoma HCT116 cells were purchased from the American Type Culture Collection (Manassas, Virginia, USA). HCT116 cells exhibit microsatellite instability due to an inactivating MLH1 mutation, contain a k-ras protooncogene mutation, and lack p53 and APC mutations. Cells were grown in RPMI-1640 medium (Invitrogen, Gaithersburg, Maryland, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin 100 U/ml, and streptomycin 100 mg/ml. Cultures were maintained at 37°C in 5% CO2.

Construction and transfection of the sense and antisense FPGS expression vector

The full length human FPGS cDNA was provided by Dr A Bognar (University of Toronto, Toronto, Canada). The full length human FPGS cDNA was subcloned into the EcoRI site of the eukaryotic expression vector pIREsneo (Clontech, Palo Alto, California, USA) containing a CMV promoter and a neomycin resistance gene expression cassette in the sense and antisense orientation to generate the sense and antisense FPGS expression vectors, respectively. Correct integration, orientation, and sequence of the sense and antisense FPGS cDNAs were confirmed by predicted fragment sizes after multiple restriction enzyme digestions and DNA sequencing. The pIREsneo vector containing the sense or antisense FPGS cDNA was stably transfected into HCT116 cells using Lipofectin (Invitrogen) according to the manufacturer’s protocol. In a separate transfection, HCT116 cells were stably transfected with empty pIREsneo vector (endogenous FPGS).

Transfected cells were incubated with 500 µg/ml of neomycin (Invitrogen) to select for cells that expressed the various constructs. After a population of cells was selected, individual clonal cell lines were isolated and expanded. Cells were maintained in complete medium supplemented with neomycin 500 µg/ml. Several (>10) clones expressing the sense and antisense FPGS cDNA and empty vector were screened at random, and two independent clones of each construct were selected for further analyses. Data from two experiments using two independent clones of each construct were similar and thus the data from one experiment are presented.

Western blot analysis

FPGS, TS, p53, and p21 protein expression was determined by standard western analysis, as described previously, using a rabbit polyclonal antibody raised against a peptide sequence spanning amino acids 275–290 of human FPGS (Zymed, San Francisco, California, USA) at a dilution of 1:200, a sheep polyclonal antibody against human TS (Rockland Immunochemicals, Gilbertsville, Pennsylvania, USA) at a dilution of 1:3000, and anti-p53(DO-1) and p21 monoclonal antibody (Oncogene Research Products, Boston, Massachusetts, USA) at a dilution of 1:3000, respectively. All western analyses were repeated using three different cell lysates.

FPGS and TS activity assay

FPGS activity was determined by measuring incorporation of [3H]glutamate into the polyglutamate chain of aminopterin, as described previously. The catalytic activity of TS was determined by 7H release that occurred during conversion of [5-3H]-deoxyuridine-5-monophosphate to deoxothymidine-5-monophosphate, as described previously. Enzyme assays were performed in triplicate and repeated using three different cell lysates.

Intracellular folate concentrations and determination of glutamate chain lengths

Intracellular folate concentrations were determined by a standard microbiological microtitre plate assay using Lactobacillus casei for both conjugate treated and untreated samples to determine the extent of polyglutamylation as L casei grow in proportion to the amount of mono-, di-, tri-, and tetra-glutamylated folate in the samples, and conjugate treatment results in cleavage of the polyglutamylated chain to yield these short chain length forms of folate. This method has been used in previous studies to determine overall pool of long chain versus short chain polyglutamates. All analyses were performed in triplicate and repeated using three different cell lysates.

Real time quantitative reverse transcription-PCR

Total cellular RNA was extracted using the RNeasy MidiKit (Qiagen, Mississauga, Ontario, Canada). cDNA was generated from total RNA using random primers and the SuperScript III RNase H-Reverse Transcriptase (Invitrogen). Polymerase chain reaction (PCR) primers for GGH were constructed based on the human GGH cDNA sequence and were synthesised by ACGT (Toronto, Ontario, Canada) as follows: forward, 5′-GCC ACA GAT ACT GTT GAC GTG G-3′; reverse, 5′-ATG GAA ATT GGC ATT CAG AGG-3′. Real time PCR was performed using the LC FastStart DNA Master SYBR Green 1 Kit in the LightCycler rapid thermal cycler system (Roche Diagnostics, Laval, Quebec, Canada) and the melting curve was determined as described. All PCR reactions were performed in triplicate, and three replicate experiments were repeated three times.

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. The growth rate constant k was derived using the equation

\[ \frac{N}{N_0} = e^{kt} \]

where \( N_0 \) is the OD of cells at time zero and \( N \) is the OD of cells at 72 hours. The same equation was used to calculate the doubling time \( t \) by setting \( N/N_0 = 2 \). All analyses were performed in triplicate, and three replicate experiments were performed.

In vitro chemosensitivity assay

In vitro chemosensitivity was determined using a modification of the SRB protein assay, as described previously. Briefly, 8000 cells per 100 µl RPMI-1640 medium per well were seeded in triplicate in 96 well flat bottom plates (Costar, Cambridge, Massachusetts, USA). After 24 hours, an additional 100 µl of RPMI-1640 medium containing MTX (Schircks, Jona, Switzerland) or 5-FU (InvivoGen, San Diego, California, USA) in combination with LV (Sigma Aldrich Canada, Oakville, Ontario, Canada) were added, and cells were cultured for an additional 72 hours. The
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The dye was solubilised, and the OD of the solution measured with trichloroacetic acid and stained with SRB protein dye.

3.5

tion of MTX was varied, with concentrations ranging from

10

6

and 25%, respectively, compared with survival of untreated cells expressing endogenous FPGS (p <= 0.0001).

Comparisons among cells expressing sense FPGS, antisense (FPGS-AS) folylpolyglutamate synthetase (FPGS), Conjugase treatment allows measurement of total folate content, including short and long chain polyglutamates, whereas non-conjugase treatment determines the content of short chain polyglutamates. Intracellular concentrations of total folate were significantly higher in cells expressing the sense FPGS and lower in cells expressing the antisense FPGS, respectively, than in those expressing endogenous FPGS (p <= 0.0001). Differences between mean folate concentration for conjugase treated and untreated samples (which allows determination of long chain polyglutamates) were significantly higher in cells expressing the sense FPGS and lower in cells expressing the antisense FPGS, respectively, compared with those expressing endogenous FPGS (p <= 0.001).

RESULTS

HCT116 cells expressing the sense and antisense FPGS had significantly higher and lower steady state levels of FPGS protein (A) and FPGS activity (B), respectively, compared with cells transfected with vector alone (VA; endogenous FPGS) (p <= 0.001).

HCT116 cells expressing the sense FPGS had an 8.2-fold higher, whereas those expressing the antisense FPGS had a 2.3-fold lower, FPGS activity compared with those expressing endogenous FPGS (p <= 0.001) (fig 1B). HCT116 cells expressing the sense and antisense FPGS grew faster and slower, respectively, than those expressing endogenous FPGS, as reflected by a significantly decreased and increased doubling time, respectively (32.0 (0.6) hours (sense) v 34.9 (0.4) hours (endogenous) v 36.4 (0.7) hours (antisense); p <= 0.001).

Following conjugase treatment (which allows measurement of total folate content, including short and long chain polyglutamates), intracellular folate concentration of HCT116 cells expressing the sense FPGS was 30% higher, whereas that of those expressing the antisense FPGS was 21% lower, compared with those expressing endogenous FPGS (p <= 0.001) (fig 2). Intracellular folate concentration of samples not treated with conjugase (which allows determination of short chain polyglutamates) was not significantly different between cells expressing the sense FPGS and those expressing endogenous FPGS and between cells expressing the antisense FPGS and cells expressing endogenous FPGS (fig 2). However, there were significantly more short chain polyglutamates in cells expressing the sense FPGS compared with those expressing the antisense FPGS.

Figure 1 HCT116 colon cancer cells transfected with the sense (FPGS-S) and antisense (FPGS-AS) folylpolyglutamate synthetase (FPGS) cDNA had significantly higher and lower steady state levels of FPGS protein (A) and FPGS activity (B), respectively, compared with cells transfected with vector alone (VA; endogenous FPGS) (p <= 0.001).

Figure 2 Intracellular folate concentrations of HCT116 cells expressing the sense (FPGS-S), endogenous (vector alone (VA)), and antisense (FPGS-AS) folylpolyglutamate synthetase (FPGS). Conjugase treatment allows measurement of total folate content, including short and long chain polyglutamates, whereas non-conjugase treatment determines the content of short chain polyglutamates. Intracellular concentrations of total folate were significantly higher in cells expressing the sense FPGS and lower in cells expressing the antisense FPGS, respectively, than in those expressing endogenous FPGS (p <= 0.0001). Differences between mean folate concentration for conjugase treated and untreated samples (which allows determination of long chain polyglutamates) were significantly higher in cells expressing the sense FPGS and lower in cells expressing the antisense FPGS, respectively, compared with those expressing endogenous FPGS (p <= 0.001).
Cells expressing the sense FPGS had significantly higher, whereas those expressing the antisense FPGS had significantly lower, GGH mRNA expression, compared with those expressing endogenous FPGS (p<0.05) (fig 3).

In vitro chemosensitivity to 5-FU plus LV was significantly different among HCT116 cells transfected with the three different FPGS constructs (p<0.001) (fig 4A). In vitro chemosensitivity of HCT116 cells expressing the sense FPGS to 5-FU plus LV was significantly enhanced compared with those expressing endogenous or the antisense FPGS (p<0.001) (fig 4A). In contrast, in vitro chemosensitivity of HCT116 cells expressing the antisense FPGS was significantly decreased compared with those expressing endogenous or the sense FPGS (p = 0.0122 and p<0.0001, respectively) (fig 4A). IC_{25}, IC_{50}, and IC_{75} values for 5-FU indicate significantly enhanced chemosensitivity of HCT116 cells expressing the sense FPGS and reduced chemosensitivity of those expressing the antisense FPGS compared with cells expressing endogenous FPGS (table 1).

Overall, there was no significant difference in in vitro chemosensitivity to MTX among HCT116 cells transfected with the three different FPGS constructs (p = 0.548) (fig 4B). IC_{25}, IC_{50}, and IC_{75} values of MTX, however, suggest that antisense FPGS inhibition enhances, whereas sense FPGS overexpression decreases, chemosensitivity of HCT116 cells to MTX compared with cells expressing endogenous FPGS (table 1).

We wished to ascertain that the observed effects on chemosensitivity of HCT116 cells to 5-FU and MTX were secondary to alterations in FPGS and not an unintended effect of FPGS modulation on intrinsic TS protein expression and activity. TS is a critical target for 5-FU and MTX, and its expression level is an important predictor of chemosensitivity of cancer cells to 5-FU and MTX.\textsuperscript{35} TS protein expression and catalytic activity were similar among HCT116 cells expressing the sense, antisense, and endogenous FPGS that were not treated with 5-FU or MTX (data not shown). The effect of FPGS modulation on two molecular determinants of chemosensitivity, p53 and p21,\textsuperscript{36–38} was also investigated. FPGS overexpression downregulated, whereas FPGS inhibition upregulated, p53 and p21 protein expression compared with endogenous FPGS (fig 5).

**Figure 3** HCT116 cells expressing the sense folicpolyglutamate synthetase (FPGS-S) had significantly higher, whereas those expressing the antisense FPGS (FPGS-AS) had significantly lower, $\gamma$-glutamyl hydrolase (GGH) mRNA expression compared with those expressing endogenous FPGS (vector alone (VA)) (p<0.05), as determined by real time quantitative reverse transcription-polymerase chain reaction. GGH is a lysosomal peptidase that removes the terminal glutamates, thereby countering the action of FPGS. Human 5-aminolevulinate delta synthase 1 was used as the endogenous reference gene (primers: forward, 5'-TGC CCA TTC TTA TCC CGA GT-3'; reverse, 5'-GGT TTC TTT GAT CTG TTG GGA GT-3'). Relative quantification was performed using LC Relative Quantification Software version 1.0 (Roche Diagnostics).

**Figure 4** (A) In vitro chemosensitivity to 5-fluorouracil (5-FU) plus leucovorin (LV) was significantly different among HCT116 colon cancer cells expressing the sense (FPGS-S), endogenous (vector alone (VA)), and antisense (FPGS-AS) folicpolyglutamate synthetase (FPGS) (p<0.001). Chemosensitivity of cells expressing the sense FPGS to 5-FU+LV was significantly enhanced compared with those expressing endogenous or the antisense FPGS (p<0.0001). In contrast, chemosensitivity of cells expressing the antisense FPGS was significantly decreased compared with those expressing endogenous or the sense FPGS (p=0.0122 and p<0.0001, respectively). (B) In vitro chemosensitivity to methotrexate (MTX) was not significantly different among HCT116 cells expressing the sense, endogenous, and antisense FPGS (p = 0.548).
DISCUSSION
We developed an in vitro model of FPGS overexpression and inhibition in HCT116 colon cancer cells with predictable functional consequences. Compared with HCT116 cells expressing endogenous FPGS, those expressing the sense FPGS had significantly higher FPGS expression and activity, higher GGH expression, faster growth rates, higher concentrations of total intracellular folate, and higher content of long chain polyglutamates. In contrast, HCT116 cells expressing the antisense FPGS had significantly lower FPGS expression and activity, lower GGH expression, slower growth rates, lower concentrations of total intracellular folate, and lower content of long chain polyglutamates compared with those expressing endogenous FPGS. These observed metabolic and functional consequences of FPGS overexpression and inhibition are consistent with the known biological function of FPGS and provided an appropriate in vitro model to test the effect of FPGS overexpression and inhibition on chemosensitivity of colon cancer cells to 5-FU and MTX.

Using this system, we have shown that FPGS overexpression enhances, whereas FPGS inhibition decreases, chemosensitivity of colon cancer cells to 5-FU plus LV. The most likely explanation for this observation is that FPGS overexpression enhanced the cytotoxic effect of 5-FU by increasing relative intracellular concentrations of longer chain length 5,10-methyleneTHF polyglutamates, resulting in more efficient formation and stabilisation of the inhibitory 5,10-methyleneTHF-TS-FdUMP ternary complex. In contrast, FPGS inhibition decreased the cytotoxic effect of 5-FU by decreasing relative intracellular concentrations of longer chain length 5,10-methyleneTHF polyglutamates, resulting in less efficient formation and stabilisation of the 5,10-methyleneTHF-TS-FdUMP ternary complex. However, we did not provide direct evidence that FPGS modulation lead to different glutamate chain lengths of 5,10-methyleneTHF because of technical difficulties in measuring intracellular 5,10-methyleneTHF. Furthermore, we did not determine and compare the rate of formation and dissociation and the concentration of the 5,10-methyleneTHF-TS-FdUMP ternary complex among HCT116 cells transfected with the three different FPGS constructs. However, indirect evidence from a prior study supports the proposed mechanism by which FPGS modulation might have affected chemosensitivity of colon cancer cells to 5-FU. In that study, the effect of LV, which is converted to 5,10-methyleneTHF and enters the folate pathway, on chemosensitivity of human leukaemia CCRF-CEM (the parent cell line with proficient polyglutamylation) and CCRF-CEM/P (a cell line with impaired ability to form polyglutamates) to 5-FU was compared. Both CCRF-CEM and CCRF-CEM/P cells accumulated 5,10-methyleneTHF in the presence of LV in a dose dependent manner. However, at a dose of 5-FU that produced only a slight decrease in cell growth, addition of LV further inhibited the cell growth in CCRF-CEM cells, but not in CCRF-CEM/P cells, suggesting that the impaired polyglutamylation of 5,10-methyleneTHF was likely responsible for the lack of potentiation of the cytotoxic effect of 5-FU by LV in CCRF-CEM/P cells.

The observed effect of FPGS modulation on chemosensitivity to 5-FU is consistent with the existing in vitro evidence that suggests that the cytotoxic effect of 5-FU is directly correlated with FPGS activity in 14 human cancer cell lines. Another in vitro study has shown that human colon cancer HCT8 cells become rapidly resistant to 5-FU over time owing to a progressive decrease in FPGS mRNA expression and activity. In contrast, three small human studies have shown conflicting results concerning the role of FPGS activity and mRNA expression in predicting treatment response to 5-FU+LV and survival in patients with colon cancer.

<table>
<thead>
<tr>
<th>IC₅₀, IC₅₀, and IC₇₅ values (that is, drug concentration that corresponded to a reduction in cell survival by 75%, 50%, and 25%, respectively, compared with the survival of untreated control cells) were calculated from plots of drug concentration versus proportion of cells that survived.</th>
<th>25% survival</th>
<th>50% survival</th>
<th>75% survival</th>
</tr>
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<tbody>
<tr>
<td>IC₅₀</td>
<td>IC₅₀</td>
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<tr>
<td>FPGS-S</td>
<td>3.90 (3.30, 4.63)</td>
<td>1.64 (1.26, 2.14)</td>
<td>0.35 (0.26, 0.47)</td>
</tr>
<tr>
<td>VA</td>
<td>17.03 (15.39, 18.83)</td>
<td>3.03 (2.71, 3.37)</td>
<td>0.54 (0.42, 0.68)</td>
</tr>
<tr>
<td>FPGS-AS</td>
<td>59.83 (48.83, 73.30)</td>
<td>4.57 (4.15, 5.02)</td>
<td>0.69 (0.47, 1.01)</td>
</tr>
</tbody>
</table>

**Table 1**

**Figure 5** Effect of folypolyglutamate synthetase (FPGS) modulation on p53 and p21. p53 is integrally involved in cell cycle control, DNA repair, and apoptosis while p21 is a member of the cyclin dependent kinase inhibitor family, which inhibits G₁ and S phase progression. p53 promotes cell cycle arrest in late G₁ through upregulation of p21 in response to cell injury, thereby allowing time for repair, whereas in the event that DNA damage is more severe and non-reparable, p53 performs its alternative role of moving the cell into apoptosis. FPGS overexpression (FPGS-S) downregulated, whereas FPGS inhibition (FPGS-AS) upregulated, p53 and p21 protein expression compared with endogenous FPGS (vector alone (VA)) in HCT116 cells (densitometry of bands: p53:β-actin ratio 1.9 [FPGS-S] v 3.1 [VA] v 4.1 [FPGS-AS]; p21:β-actin ratio 1.7 [FPGS-S] v 2.5 [VA] v 3.2 [FPGS-AS]).
Large clinical trials are therefore necessary to investigate the potential clinical utility of FPGS status as a predictor of treatment response and a prognostic indicator in colon cancer patients receiving 5-FU based chemotherapy.

FPGS modulation had no significant overall effect on the chemosensitivity of colon cancer cells to MTX, in contrast with our hypothesis that FPGS induced changes in the glutamate chain lengths of MTX would affect chemosensitivity of colon cancer cells to MTX by altering intracellular MTX retention and affinity of MTX for its target folate dependent enzymes in the thymidylate and purine biosynthetic pathways. However, the overall pattern of chemosensitivity to MTX and the IC50, IC50, and IC75 values of MTX suggest that FPGS inhibition may enhance, whereas FPGS overexpression may decrease, chemosensitivity of colon cancer cells to MTX. Our data contrast with prior observations, which suggested that FPGS downregulation leads to resistance, whereas FPGS overexpression enhances chemosensitivity to MTX and novel antifolates in other cancer cell lines.12–18 It is possible that our results are specific to the cell type studied because there are tissue specific differences in MTX metabolism and in FPGS expression and activity.1,2 Also, colon cancer cells are generally not sensitive to MTX.42 Another possible explanation is that the effect of the FPGS modulation induced changes in the glutamate chain lengths of MTX might have been nullified by similar changes in the glutamate chain lengths of intracellular folate induced by FPGS modulation.43–47

We have found that FPGS overexpression downregulates, whereas FPGS inhibition upregulates, p53 and p21 expression in untreated HCT116 cells. Whether or not FPGS modulation induced p53 and p21 changes played a role in altering chemosensitivity of HCT116 cells to 5-FU was not determined in the present study. Given the very small magnitude of changes in p53 and p21 protein expression, however, it is highly unlikely that they contributed significantly to altered chemosensitivity. Nevertheless, the direction of changes of p53 and p21 is consistent with the observed alterations in intracellular folate concentrations resulting from FPGS modulation (that is, the effect of intracellular folate level on DNA damage and consequent p53 activation and p21 upregulation48–50).

At present, mechanisms by which FPGS expression and activity are decreased or qualitatively altered in cancer cells resistant to MTX and 5-FU51–53 are not clearly elucidated. To date, only one putative mutation in FPGS has been identified.54 Therefore, it is of great interest to elucidate genetic, epigenetic, and other potential mechanisms of FPGS regulation in tumours.

In conclusion, our study provides evidence that FPGS overexpression enhances, whereas FPGS inhibition decreases, chemosensitivity of colon cancer cells to 5-FU. Our data suggest that FPGS status may be an important determinant, and hence a useful clinical predictor, of the chemosensitivity of colon cancer cells to 5-FU based chemotherapy. Furthermore, our data provide, for the first time, evidence suggesting that FPGS gene transfer may be a potential target for increasing sensitivity of colon cancer cells to 5-FU based chemotherapy. In contrast, FPGS modulation appears to have no significant effect on the chemosensitivity of colon cancer cells to MTX. However, our data based on a single colon cancer cell line need to be confirmed in other colon cancer cell lines.

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