Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 29 (2016) 27–35

Journal of Nutritional Biochemistry

Effects of folylpolyglutamate synthase modulation on global and gene-specific DNA methylation and gene expression in human colon and breast cancer cells

Sung-Eun Kim[a,b,*], Toshinori Hinoue[c], Michael S. Kim[b], Kyoung-Jin Sohn[b,d], Robert C. Cho[a], Daniel J. Weisenberger[e,f], Peter W. Laird[c], Young-In Kim[a,b,d,g]

[a]Department of Nutritional Sciences, University of Toronto, Toronto, ON, Canada M5S 1A8
[b]Keenan Research Centre for Biomedical Science of St. Michael's Hospital, Toronto, ON, Canada M5B 1T8
[c]Center for Epigenetics, Van Andel Research Institute, Grand Rapids, MI 49503-2518, USA
[d]Department of Medicine, University of Toronto, Toronto, ON, Canada M5S 1A8
[e]USC Epigenome Center, University of Southern California, Los Angeles, CA 90089-9601, USA
[f]Department of Biochemistry and Molecular Biology, University of Southern California, Los Angeles, CA 90089-9601, USA
[g]Division of Gastroenterology, St. Michael's Hospital, Toronto, ON, Canada M5B 1W8

Received 17 June 2015; received in revised form 10 October 2015; accepted 27 October 2015

Abstract

Folylpolyglutamate synthase (FPGS) plays a critical role in intracellular folate homeostasis. FPGS-induced polyglutamylated folates are better substrates for several enzymes involved in the generation of *S*-adenosylmethionine, the primary methyl group donor, and hence FPGS modulation may affect DNA methylation. DNA methylation is an important epigenetic determinant in gene expression and aberrant DNA methylation is mechanistically linked cancer development. We investigated whether FPGS modulation would affect global and gene-specific promoter DNA methylation with consequent functional effects on gene expression profiles in HCT116 colon and MDA-MB-435 breast cancer cells. Although FPGS modulation altered global DNA methylation and DNA methyltransferases (DNMT) activity, the effects of FPGS modulation on global DNA methylation and DNMT activity could not be solely explained by intracellular folate concentrations and content of long-chain folylpolyglutamates, and it may be cell-specific. FPGS modulation influenced differential gene expression and promoter cytosine-guanine dinucleotide sequences (CpG) DNA methylation involved in cellular development, cell cycle, cell death and molecular transport. Some of the altered gene expression was associated with promoter CpG DNA methylation changes. In both the FPGS-overexpressed HCT116 and MDA-MB-435 cell lines, we identified several differentially expressed genes involved in folate biosynthesis and one-carbon metabolism, which might in part have contributed to the observed increased efficacy of 5-fluorouracil in response to FPGS overexpression. Our data suggest that FPGS modulation affects global and promoter CpG DNA methylation and expression of several genes involved in important biological pathways. The potential role of FPGS modulation in DNA methylation and its associated downstream functional effects warrants further studies.
© 2015 Elsevier Inc. All rights reserved.

Keywords: Folate; Folylpolyglutamate synthase (FPGS); DNA methylation; Gene expression; Colon cancer; Breast cancer

1. Introduction

Folate plays an essential role in nucleotide biosynthesis and biological methylation reactions as an important mediator of one-carbon transfer reactions [1,2]. Monoglutamylated folates are the only circulating form of folate in blood and the only form of folate transported across the cell membrane [2]. Once taken up into cells, however, intracellular folates exit primarily as polyglutamylated forms [2]. Polyglutamylated folates are better retained in cells and are better substrates for folate-dependent enzymes compared with monoglutamylated counterparts [3,4]. Intracellular polyglutamylation of folate is regulated by folylpolyglutamate synthase (FPGS), which induces polyglutamylation [2]. In addition, FPGS plays an important role in antifolate polyglutamylation. Polyglutamylated antifolates are retained in cells longer, thereby increasing their cytotoxicity by extending the length of exposure [2–4]. Furthermore, polyglutamylated antifolates have a higher affinity for and, hence, inhibit their target folate-dependent enzymes in nucleotide biosynthesis to a greater extent than the monoglutamylated forms [3,4]. FPGS, along with a tightly coupled counterregulation by γ-glutamyl hydrolase (GGH), which catalyzes the hydrolysis of polyglutamylated folate into monoglutamates and facilitates the export of folate out of cells, plays an important role in maintenance of optimal intracellular concentrations and polyglutamylation of folates and antifolates. Dysregulation and

Abbreviations: CpG, cytosine-guanine dinucleotide sequences; DNMT, DNA methyltransferases; FPGS, folylpolyglutamate synthase; 5FU, 5-fluorouracil; GGH, γ-glutamyl hydrolase; MRP5, multidrug-resistance-associated protein 5; qRT-PCR, quantitative reverse-transcriptase PCR; SAM, *S*-adenosylmethionine; siRNA, small-interfering RNA.

* Corresponding author. Room 516, Keenan Research Center for Biomedical Science of St. Michael's Hospital, 209 Victoria Street, Toronto, ON, Canada M5B 1T8. Tel.: +1-416-847-1750; fax: +1-416-864-5994.
E-mail addresses: sungeun.kim@alum.utoronto.ca (S.-E. Kim).

http://dx.doi.org/10.1016/j.jnutbio.2015.10.019

aberrancies of FPGS may contribute to the development of intracellular folate deficiency or excess-mediated diseases such as cancer and to alterations in antifolate-based cytotoxicity [3,4].

Folate, in the form of 5-methyltetrahydrofolate, participates in remethylation of homocysteine to methionine, which is a precursor of *S*-adenosylmethionine (SAM), the primary methyl group donor for most biological methylation reactions including DNA methylation [5,6]. A large body of evidence suggests that folate deficiency and excess can modulate DNA methylation in a cell, gene and site-specific manner [6]. DNA methylation is a dynamic process between active methylation, mediated by DNA methyltransferases (DNMT) using SAM as a methyl donor, and removal of methyl groups from 5-methylcytosine residues by both passive and active mechanisms [7,8]. DNA methylation of cytosine in the cytosine-guanine dinucleotide sequences (CpG) is an important epigenetic modification for gene expression and genomic stability [7]. DNA methylation occurring primarily in the bulk of the genome where CpG density is low contributes to correct organization of chromatin in active and inactive states [7]. In contrast, DNA methylation occurring in CpG rich areas clustered in small stretches of DNA termed "CpG islands", which span the 5′ end of approximately half of the human genes including the promoter and exon 1, is associated with transcriptional silencing with few exceptions [7,9].

FPGS-mediated polyglutamylation-induced changes in total intracellular folate concentrations and in contents of polyglutamylated folates may play an important role in DNA methylation as polyglutamylated folates are better substrates for methylenetetrahydrofolate reductase and methionine synthase, both of which are involved in the generation of SAM [1,3,10]. Recently, FPGS1-mediated polyglutamylated folates were found to be involved in chromatin silencing by maintaining global DNA methylation and histone H3K9 dimethylation in *Arabidopsis* [11]. Furthermore, we have recently shown that modulation of GGH, the counterregulatory enzyme of FPGS, is associated with functionally significant DNA methylation alterations in several important biological pathways [12]. Aberrant or dysregulation of DNA methylation is mechanistically related to development and progression of cancer and also affects the response and toxicity of chemotherapy [7]. As such, FPGS modulation-mediated alterations in DNA methylation may have significant implication in cancer development and treatment via its functional effects on gene expression and genomic stability [6]. Using a novel *in vitro* model of FPGS overexpression and inhibition in colon and breast cancers, two of the commonest cancers globally, with predictable functional consequences [13,14], we tested the hypothesis that FPGS modulation would affect global and gene-specific DNA methylation with consequent functional effects on gene expression.

2. Materials and methods

2.1. In vitro model of FPGS overexpression and inhibition

We have previously developed and functionally characterized an *in vitro* model of FPGS overexpression and inhibition in HCT116 colon and MDA-MB-435 breast cancer cells [13,14]. FPGS overexpression was generated by transfecting both cells with the sense FPGS cDNA, whereas FPGS inhibition was developed by transfecting HCT116 and MDA-MB-435 cells with the antisense FPGS cDNA and the FPGS-targeted small-interfering RNA (siRNA), respectively [13,14]. Cells overexpressing FPGS showed significantly higher FPGS protein expression and activity, higher total intracellular folate concentrations and higher content of long-chain folylpolyglutamates compared with controls expressing endogenous FPGS [13,14]. In contrast, cells in which FPGS is inhibited had significantly lower FPGS protein expression and activity, lower concentrations of total intracellular folate and lower content of long-chain folylpolyglutamates compared with controls expressing endogenous FPGS [13,14]. The observed functional characteristics of FPGS overexpression and inhibition in this model were consistent with the known biological function of FPGS, thereby providing an appropriate *in vitro* model to test the effects of FPGS modulation on DNA methylation and gene expression [13,14]. Cells were grown in RPMI-1640 medium (Invitrogen) containing the standard concentration of folic acid (2.3 μmol/L) supplemented with 10% fetal bovine serum, 500 μg/ml Geneticin, 50 units/ml penicillin with 50 μg/ml streptomycin and 0.25 μg/ml fungizone amphotericin B. Physiological concentrations of folate *in vivo* are in nanomolar range. However, the actual physiological concentrations of folate necessary for optimal growth of immortalized transformed cells *in vitro* are likely significantly different from those for nontransformed cells *in vivo*. We have previously grown HCT116 and MDA-MB-435 cells in the same RPMI-1640 media containing nanomolar range of folic acid and observed significant growth retardation associated with 20, 50, 100 and 500 nM of folic acid in the media (data not shown). As such, cells were grown in the medium containing the standard folic acid concentration, 2.3 μmol/L, for the genomic and epigenomic analyses in order to avoid any effect associated with growth retardation in the present study. Cell cultures were maintained at 37°C in 5% CO_2. All cells were cultured for the same passage numbers (8–10 depending on the cell line and FPGS overexpression or inhibition), harvested at 80% confluence and processed for subsequent analyses.

2.2. Global DNA methylation analysis

Total genomic DNA was extracted by a standard technique using proteinase K followed by organic extraction [15]. Global DNA methylation was determined by the *in vitro* methyl acceptance assay using [^{3}H-methyl]SAM (New England Nuclear) as a methyl donor and a prokaryotic CpG DNMT, Sss1 (New England Biolabs), as previously described [12]. The manner in which this assay is performed produces an inverse relationship between the endogenous DNA methylation status and exogenous [^{3}H-methyl] incorporation. All analyses were performed in quadruplicate and repeated using two independent cell lysates.

2.3. DNMT activity assay

Total cellular CpG DNMT activity was measured by incubating cell lysate containing 10 μg of protein with 0.5 μg of poly[d(I-C)·d(I-C)] template (Sigma-Aldrich), 3 μCi [^{3}H]SAM (New England Nuclear) and lysis buffer in a total volume of 20 μl for 2 h at 37°C as previously described [12]. Each reaction was performed in triplicate and the assay was repeated three times.

2.4. Gene-specific promoter CpG DNA methylation analysis

The Illumina Infinium HumanMethylation27 (HM27) BeadChip (Illumina) was performed to interrogate the DNA methylation status of 27,578 individual CpG sites located at promoter regions of 14,495 genes as previously described [12,16]. A measure of the level of DNA methylation at each CpG site was scored as beta (β) values. DNA methylation β-values represent the ratio of the intensity of the methylated bead type to the combined locus intensity ranging from 0 to 1. Values close to 0 indicate low levels of DNA methylation, while values close to 1 indicate high levels of DNA methylation [16]. Statistical analysis and data visualization were carried out using the R/Bioconductor software packages (http://www.bioconductor.org).

DNA hypermethylation or hypomethylation was calculated for each HM27 probe by subtracting the β-value of the corresponding control from the β-value of cells expressing the sense FPGS cDNA (*Overexpression*) or cells transfected with the antisense FPGS cDNA or the FPGS-targeted siRNA (*Inhibition*). We determined the β-value difference of 0.2 as having 99% confidence based on intraassay and interassay variations [16].

2.5. Gene expression analysis

Biotinylated cRNA generated from the samples was hybridized onto the Illumina HumanHT-12 v4.0 BeadChip (Illumina) as previously described [12]. Each array on this BeadChip targets 31,335 annotated genes and includes 47,231 probes. After washing and staining, each BeadChip was scanned on the iScan (Illumina), and the intensity files were quantified in GenomeStudio (Illumina) to generate intensity measurements without normalization algorithms. Normalization and data filtering were performed separately for each subset of samples being used in a particular analysis. An unpaired *t* test using a false discovery rate Benjamini and Hochberg multiple testing correction with a *P* value cutoff of .05 was performed in order to compare between cells expressing the sense FPGS cDNA and the corresponding control (*Overexpression*) and between cells transfected with the antisense FPGS cDNA or the FPGS-targeted siRNA and the corresponding control (*Inhibition*).

2.6. Integrated analysis of DNA methylation and gene expression data

We merged the DNA methylation and gene expression data sets using Entrez Gene IDs for the integrated analysis to identify genes, differential expression of which was regulated by DNA methylation in response to FPGS modulation. We used a β-value difference ($|\Delta\beta|$) of 0.20 as a threshold for differential DNA methylation between cells in which FPGS was overexpressed or inhibited and the corresponding control. This threshold of $|\Delta\beta|=0.20$ was determined previously as a stringent estimate of Δβ detection sensitivity across the range of β-values [16]. Gene expression data with (1) a fold change greater or less than 1.3 and (2) a one-way ANOVA with a Benjamini and Hochberg corrected *P* value ≤.05 were used for integrated analysis. We set 1.3 as a fold change not to overlook small changes in response to FPGS modulation as we identified relatively a small number of genes differentially expressed especially in HCT116 cells with FPGS inhibition.

2.7. Functional analysis

The functional analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems; http://www.ingenuity.com) to identify biological functions that were most significant to genes differentially methylated and/or expressed in each system. The right-tailed Fisher's Exact Test was used to calculate *P* values in determining the probability that each biological function assigned to that data set is due to chance alone.

2.8. Validation of gene expression data

Quantitative reverse-transcriptase PCR (qRT-PCR) was performed to confirm the gene expression data obtained using the Illumina HumanHT-12 v4.0 BeadChip as previously described [12]. Selected primer sequences (Integrated DNA Technologies) are presented in Supplementary Table 1. Relative gene expression data were analyzed using the comparative threshold method [17].

2.9. Statistical analysis

Differences in global DNA methylation, DNMT activity and qRT-PCR data between cells overexpressing FPGS and the corresponding control and between cells with FPGS inhibition and the corresponding control were analyzed using the Student's *t* test function of SPSS Statistics 17.0 (IBM SPSS, Chicago, IL). The results were considered statistically significant if two-tailed *P* values were <.05.

3. Results

3.1. Effects of FPGS modulation on global DNA methylation and DNMT activity

FPGS overexpression was associated with significantly lower (by 18%) global DNA methylation than controls in HCT116 cells ($P<.001$; Fig. 1A) but was associated with significantly higher (by 13%) global DNA methylation than controls in MDA-MB-435 cells ($P<.001$; Fig. 1B). FPGS inhibition was associated with significantly higher (by 12%) global DNA methylation than controls in HCT116 cells ($P=.003$; Fig. 1A) but had no effect in MDA-MB-435 cells ($P=.59$; Fig. 1C).

FPGS overexpression was associated with lower (by 77%) DNMT activity than controls in HCT116 cells ($P<.001$; Fig. 1D) but was associated with significantly higher (by 115%) DNMT activity than controls in MDA-MB-435 cells ($P<.001$; Fig. 1E). FPGS inhibition was associated with significantly lower DNMT activity than controls expressing endogenous FPGS in both HCT116 (by 31%, $P=.017$; Fig. 1D) and MDA-MB-435 (by 64%, $P<.001$; Fig. 1F) cells.

3.2. Effect of FPGS modulation on gene-specific CpG promoter DNA methylation

In HCT116 cells, we identified 864 differentially methylated genes (446 hypermethylated and 418 hypomethylated) in response to FPGS overexpression and 626 differentially methylated genes (247 hypermethylated and 379 hypomethylated) in response to FPGS inhibition. Major function categories of the biological and disease processes affected by the FPGS modulation-induced differentially methylated genes included cellular assembly and organization, cellular function and maintenance and cellular movement in the FPGS-overexpressed HCT116 cells and cellular movement in the FPGS-inhibited HCT116 cells (Supplementary Table 2).

In MDA-MB-435 cells, we identified 2239 differentially methylated genes (1161 hypermethylated and 1078 hypomethylated) in response to FPGS overexpression and 2024 differentially methylated genes (1150 hypermethylated and 874 hypomethylated) in response to FPGS inhibition. Differentially methylated genes were involved in molecular transport and cell death in both the FPGS-overexpressed and inhibited MDA-MB-435 cells (Supplementary Table 2).

3.3. Effect of FPGS modulation on gene expression

In HCT116 cells, we identified 2897 differentially expressed genes (1576 downregulated and 1321 upregulated) in response to FPGS overexpression and 359 differentially expressed genes (129 downregulated and 230 upregulated) in response to FPGS inhibition. Genes involved in cell cycle, cellular assembly and organization, DNA replication, recombination, and repair, RNA posttranscriptional

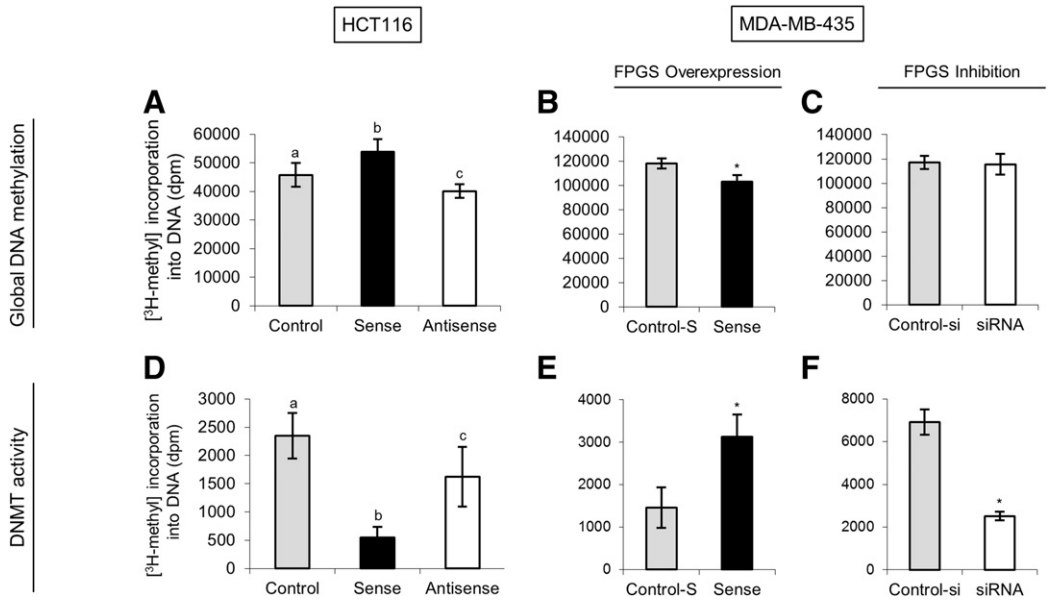

Fig. 1. Effect of FPGS modulation on global DNA methylation and DNMT activity in HCT116 and MDA-MB-435 cells. The *in vitro* methyl acceptance assay for global DNA methylation measurement produces an inverse relationship between the endogenous DNA methylation status and exogenous [^{3}H-methyl] incorporation into DNA. All analyses were performed in quadruplicate and repeated using two independent cell lysates (A–C). The assay for DNMT activity produces a positive relationship between the endogenous enzyme activity and exogenous [^{3}H-methyl] incorporation into DNA. Each reaction was performed in triplicate and the assay was repeated three times (D–F). Analyses of HCT116 colon cancer cells are described in (A) and (D), while the analyses for MDA-MB-435 breast cancer cells are described in (B), (C), (E) and (F). *Control(-S)*, cells expressing endogenous FPGS; *Sense*, cells transfected with the sense FPGS cDNA; *Antisense*, cells transfected with the antisense FPGS cDNA; *Control-si*, cells expressing endogenous FPGS; *siRNA*, cells transfected with the FPGS-targeted siRNA. Different letters among each group denote significant difference at $P<.05$. *$P<.001$ compared with corresponding control by the Student's *t* test. Values are mean±S.D.

modification and cell death were differentially expressed in the FPGS-overexpressed HCT116 cells, while genes with functions associated with cell death, cell cycle, cell morphology, cellular function and maintenance and cellular compromise were differentially expressed in the FPGS-inhibited HCT116 cells. The list of top 50 genes most differentially expressed in the FPGS-modulated HCT116 cells is presented in Supplementary Table 3.

In MDA-MB-435 cells, we identified 1502 differentially expressed genes (840 downregulated and 662 upregulated) in response to FPGS overexpression and 829 differentially expressed genes (442 downregulated and 387 upregulated) associated with FPGS inhibition. Genes participating in cell death, cellular movement, cellular growth and proliferation, cell cycle and cell-to-cell signaling and interaction were differentially expressed in the MDA-MB-435 cells overexpressing FPGS. Genes related to cell death, cellular assembly and organization, cell cycle, cellular compromise and cellular function and maintenance were differentially expressed in the FPGS-inhibited MDA-MB-435 cells. The list of top 50 genes most differentially expressed in the FPGS-modulated MDA-MB-435 cells is presented in Supplementary Table 3.

3.4. Integrated analysis of gene expression and DNA methylation changes

We performed the integrated analysis of differentially expressed and methylated genes associated with FPGS modulation to identify genes whose expression was influenced by promoter DNA methylation changes. The list of genes with altered promoter DNA methylation and expression in the FPGS-modulated HCT116 and MDA-MB-435 cell lines is presented in Supplementary Tables 4.1–4.4.

In the FPGS-overexpressed HCT116 cells, 34 hypermethylated and downregulated genes and 31 hypomethylated and upregulated genes were identified and they were primarily involved in cell cycle, cell death and cell-to-cell signaling and interaction (Table 1 and Fig. 2). In the HCT116 cells with FPGS inhibition, we detected 2 hypermethylated and downregulated genes and 4 hypomethylated and upregulated genes (Table 1 and Fig. 2). The list of top networks matched by the genes with altered expression and promoter DNA methylation in the FPGS-modulated HCT116 cells is presented in Supplementary Table 5.1.

In the MDA-MB-435 cells overexpressing FPGS, 41 hypermethylated and downregulated genes were primarily associated with drug metabolism, molecular transport, cell cycle, cell death and cellular assembly and organization, while 54 hypomethylated and upregulated genes were mainly involved in cellular movement, cell-to-cell signaling and interaction, cell death, posttranslational modification and cell signaling (Table 1 and Fig. 2). In the FPGS-inhibited MDA-MB-435 cells, 30 downregulated and 13 upregulated genes with an inverse association with promoter DNA methylation changes were related to cellular movement and cell cycle (Table 1 and Fig. 2). The list of top networks matched by the genes with altered expression and promoter DNA methylation in the FPGS-modulated MDA-MB-435 cells is presented in Supplementary Table 5.2.

3.5. Validation of gene expression by qRT-PCR

We validated the gene expression results using qRT-PCR. We selected genes, expression of which was inversely associated with promoter DNA methylation alterations, based on the magnitude of fold change in gene expression identified from microarray analysis and relevant biological functions of interest including cancer, folate pathway, cell cycle and apoptosis. Although the magnitude of change was different, the direction of change in gene expression in response to FPGS modulation was consistent between the microarray and qRT-PCR analyses in both cell lines ($P<.05$) (Supplementary Table 6), thereby validating the microarray data.

3.6. FPGS-specific gene expression analysis

We identified genes differentially expressed in the opposite direction between FPGS overexpression and inhibition in order to determine genes whose altered expression might be FPGS modulation-specific. In HCT116 cells, 24 genes were upregulated in response to FPGS overexpression and downregulated in response to FPGS inhibition, and these genes were associated with gene expression, cell-to-cell signaling and interaction, cell morphology, cellular assembly and organization and cell death (Fig. 3A). Twenty-one genes that were downregulated in response to FPGS overexpression and upregulated in response to FPGS inhibition in HCT116 cells were involved in cell cycle, cellular compromise, lipid metabolism, small-molecule biochemistry and vitamin and mineral metabolism (Fig. 3A). The top 10 genes associated with the FPGS-specific altered expression in the FPGS-modulated HCT116 cells are shown in Table 2. The list of top networks matched by the genes associated with the FPGS-specific altered expression in the FPGS-modulated HCT116 cells is presented in Supplementary Table 7. We identified one gene with FPGS-specific altered expression changes that appeared to be regulated by promoter DNA methylation in HCT116 cells. *ALDH1A3* was downregulated in

Table 1
The number and the top molecular and cellular functions of genes associated with altered expression and promoter DNA methylation in the FPGS-modulated HCT116 and MDA-MB-435 cells.

	HCT116		MDA-MB-435	
	Overexpression	Inhibition	Overexpression	Inhibition
Hypermethylated+Downregulated/Downregulated	34/1576	2/129	41/840	30/442
Top Functions	Cell cycle DNA replication, recombination and repair Cell death Cell morphology Cell-to-cell signaling and interaction	Gene expression Cellular function and maintenance	Drug metabolism Molecular transport Cell cycle Cell death Cellular assembly and organization	Cellular movement Carbohydrate metabolism Small-molecule biochemistry Cellular growth and proliferation Cell cycle
Hypomethylated+Upregulated/Upregulated	31/1321	4/230	54/662	13/387
Top Functions	Cell death Cell cycle Cell-to-cell signaling and interaction Cellular development Cellular function and maintenance	Drug metabolism Molecular transport Lipid metabolism Small-molecule biochemistry Amino acid metabolism	Cellular movement Cell-to-cell signaling and interaction Cell death Posttranslational modification Cell signaling	Cell cycle Cellular movement Posttranslational modification Protein degradation Protein synthesis

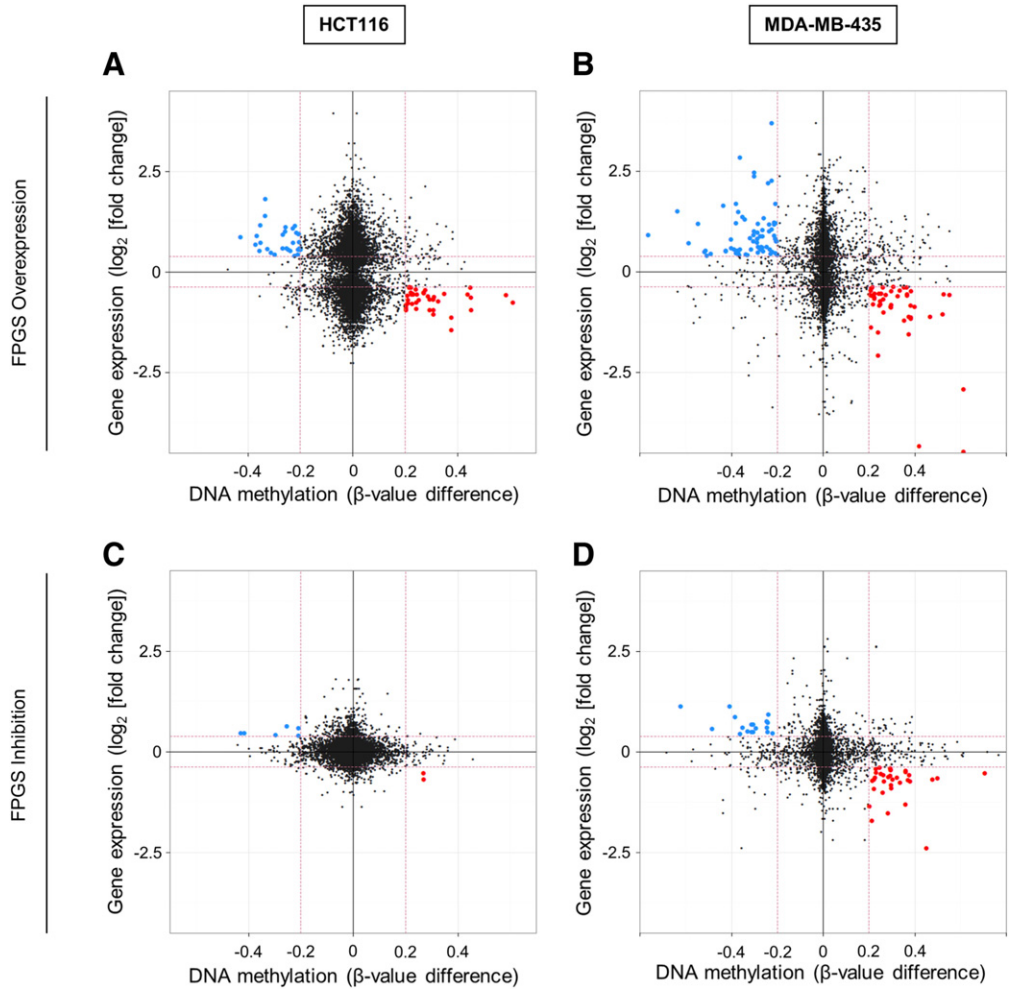

Fig. 2. Integrated analysis of gene expression and promoter DNA methylation changes in the FPGS-modulated HCT116 and MDA-MB-435 cells. FPGS overexpression is described in (A) and (B), while FPGS inhibition is depicted in (C) and (D). HCT116 cells are analyzed in (A) and (C), while MDA-MB-435 cells are analyzed in (B) and (D). The β-value difference and $\log_2$-transformed gene expression value difference between *Sense* and the corresponding control (*Control*, HCT116; *Control-S*, MDA-MB-435) and between *Antisense/siRNA* and the corresponding control (*Control*, HCT116; *Control-si*, MDA-MB-435) are plotted on *x*- and *y*-axes, respectively. Red data points highlight those genes that are hypermethylated with β-value difference of >0.2 and show less than −1.3 fold change in their expression levels, while blue data points indicate those genes that are hypomethylated with β-value difference of less than −0.2 and show >1.3 fold change in their expression levels.

response to FPGS overexpression (fold change: −1.61) and upregulated in response to FPGS inhibition (fold change: 1.85) and was hypermethylated in the FPGS-overexpressed HCT116 cells (β-value difference: 0.29) (Table 2 and Supplementary Table 4).

In MDA-MB-435 cells, we identified 191 genes that were upregulated in response to FPGS overexpression and downregulated in response to FPGS inhibition, and these genes were associated with cell cycle, gene expression, cell death, cellular growth and proliferation and cell-to-cell signaling and interaction (Fig. 3B). One hundred twenty-two genes involved in lipid metabolism, small-molecule biochemistry, carbohydrate metabolism, molecular transport and nucleic acid metabolism were downregulated in response to FPGS overexpression and upregulated in response to FPGS inhibition in MDA-MB-435 cells (Fig. 3B). The top 10 genes associated with the FPGS-specific altered expression in the FPGS-modulated MDA-MB-435 cells are shown in Table 3. The list of top networks matched by the genes associated with the FPGS-specific altered expression in the FPGS-modulated MDA-MB-435 cells is presented in Supplementary Table 7.

We also identified several genes with FPGS-specific altered expression changes that might have been regulated by promoter DNA methylation in MDA-MB-435 cells (Table 3 and Supplementary Table 4). *HLA-DPA1* (major histocompatibility complex, class II, DP alpha 1) was upregulated and hypomethylated (fold change: 2.82, β-value difference: −0.64) in response to FPGS overexpression, while it was downregulated and hypermethylated (fold change: −2.87, β-value difference: 0.28) in response to FPGS inhibition. *THBS2* (thrombospondin 2) and *C1S* (complement component 1, s subcomponent) were downregulated and hypermethylated (*THBS2*, fold change: −5.25, β-value difference: 0.45; *C1S*, fold change: −3.26, β-value difference: 0.21) in response to FPGS inhibition, while they were upregulated (*THBS2*, fold change: 2.13; *C1S*, fold change: 1.59) in response to FPGS overexpression. *PLSCR1* was associated with downregulation and hypermethylation (fold change: −2.62, β-value difference: 0.21) in response to FPGS overexpression, whereas it was upregulated (fold change: 1.97) in response to FPGS inhibition.

4. Discussion

FPGS plays a pivotal role in maintenance of optimal intracellular concentrations and polyglutamylation of folate for important folate-mediated one-carbon transfer reactions involved in nucleotide biosynthesis and biological methylation reactions including DNA methylation [2]. Intracellular folate depletion/excess and altered folylpolyglutamate distribution leads to perturbations in the

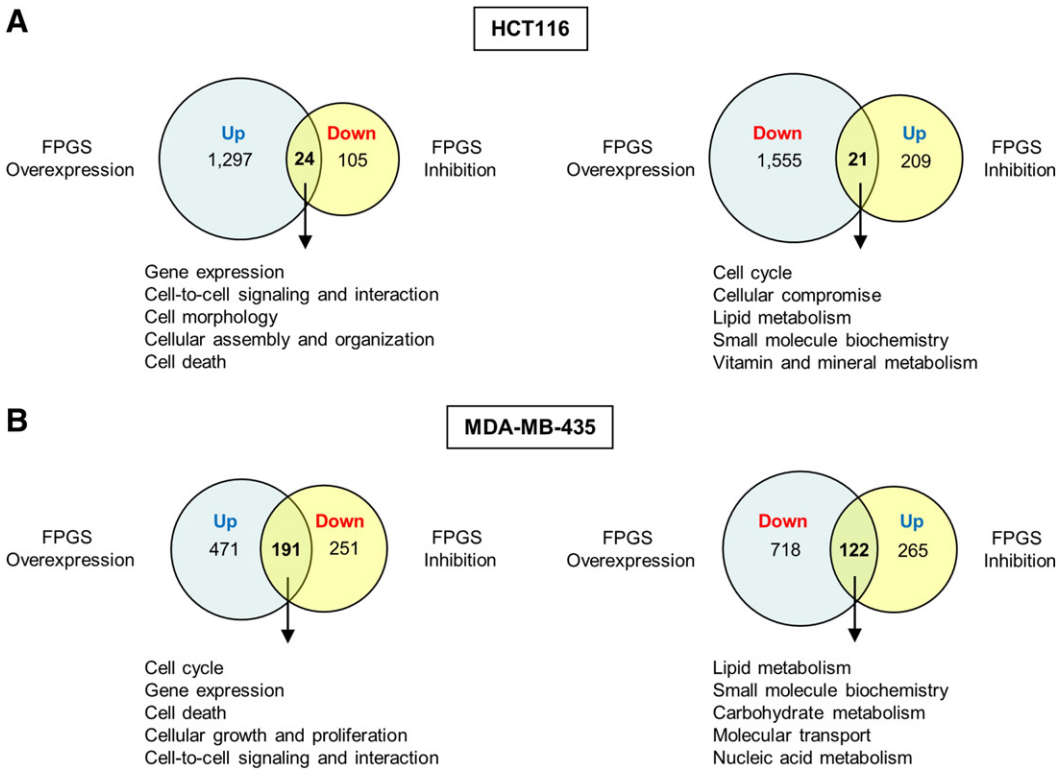

Fig. 3. The number and the top molecular and cellular functions of genes associated with the FPGS-specific altered expression. Analyses of HCT116 colon cancer cells are described in (A), while the analyses for MDA-MB-435 breast cancer cells are described in (B).

nucleotide synthesis and biological methylation pathways [2,18]. Indeed, folate deficiency has been linked to the development of several human diseases including cancers, primarily through aberrant DNA synthesis, stability, integrity, repair and methylation [1,5]. Furthermore, folate excess has been shown to exert adverse health effects, including tumor promotion, likely via aberrant nucleotide biosynthesis and biological methylation reactions [1,19]. Given the essential role of FPGS in intracellular folate homeostasis, dysregulation of FPGS and consequent perturbations in intracellular folate concentrations and folylpolyglutamate distribution will likely be associated with aberrant folate and one-carbon metabolism, thereby contributing to the development and progression of certain human diseases that are linked to folate deficiency and excess. Based on the observations that polyglutamylated folates are better substrates for critical enzymes involved in the generation of SAM [1,3,10], we posited that FPGS modulation would affect global and gene-specific promoter CpG DNA methylation with consequent functional ramifications including altered gene expression.

Our *a priori* hypothesis was that FPGS overexpression would increase global DNA methylation and DNMT activity due to the observed increased intracellular folate concentrations and higher content of long-chain folylpolyglutamates [13,14]. We observed the effect of FPGS overexpression consistent with our hypothesis in MDA-MB-435 cells. Unexpectedly, however, the effect of FPGS overexpression on global DNA methylation and DNMT activity was opposite to our hypothesis in HCT116 cells. We also hypothesized that FPGS inhibition would decrease global DNA methylation and DNMT activity due to the observed decreased intracellular folate concentrations and lower content of long-chain folylpolyglutamates [13,14]. Interestingly, although the effect of FPGS inhibition on DNMT activity was consistent with our hypothesis, its effect on global DNA methylation was not in agreement with our expectation. These data collectively suggest that the effects of FPGS modulation on global DNA methylation and DNMT activity cannot be solely explained by the observed changes in intracellular folate concentrations and content of long-chain folylpolyglutamates specific to FPGS modulation. To this end, the effect of FPGS modulation on its counterregulatory enzyme, GGH, might have influenced the FPGS modulation-induced changes in global DNA methylation and DNMT activity. In fact, the direction of change in global DNA methylation in response to FPGS overexpression and inhibition in HCT116 and MDA-MB-435 cells in the present study was more consistent with that observed with changes in GGH in response to FPGS modulation [12–14]. These observations suggest that the inverse relationship between GGH status and global DNA methylation appears to be an overriding influence on that between FPGS status and global DNA methylation.

Epigenomic and genomic analyses data reveal that FPGS modulation affected promoter CpG DNA methylation and gene expression involved in several important biological pathways. Functional analysis of differentially methylated genes revealed that, in both cell lines, genes involved in cellular development were affected by FPGS overexpression, while genes with functions relating to cell cycle and cell morphology were influenced by FPGS inhibition. In both cell lines, genes associated with cell death and molecular transport were differentially expressed in response to FPGS overexpression and inhibition, respectively. However, it is possible that some of the pathways affected by FPGS modulation might be indirect consequences of changes in total folate content and polyglutamylated folate cofactors or of changes in cell death and molecular transport. We identified only a few genes displaying the inverse association between promoter CpG methylation and gene expression in response to FPGS modulation [12,20–22]. This observation suggests that the gene expression changes in response to FPGS modulation in the present study are likely attributable to not only promoter CpG DNA methylation but also other epigenetic mechanisms such as histone modification, chromatin remodeling and RNA interference. Furthermore,

Table 2
List of the top genes associated with the FPGS-specific altered expression in the FPGS-modulated HCT116 colon cancer cells.

Gene Symbol	Fold Change (vs. Control)		Description	Accession
	FPGS Overexpression	FPGS Inhibition		
Upregulated in FPGS Overexpression and Downregulated in FPGS Inhibition				
DUSP6[a]	2.63	−1.71	Dual specificity phosphatase 6	NM_022652.2
ENC1	1.41	−1.64	Ectodermal-neural cortex (with BTB-like domain)	NM_003633.1
NFIB	1.42	−1.61	Nuclear factor I/B	NM_005596.2
PPAP2B	1.33	−1.52	Phosphatidic acid phosphatase type 2B	NM_003713.3
LGALS3	2.10	−1.48	Lectin, galactoside binding, soluble, 3 (galectin 3)	NM_002306.1
LAMB1	2.48	−1.44	Laminin, beta 1	NM_002291.1
JARID2	1.35	−1.44	Jumonji, AT-rich interactive domain 2	NM_004973.2
LOC400986	1.39	−1.44	PREDICTED: protein immunoreactive with anti-PTH polyclonal antibodies	XM_001126815.1
TANK	1.32	−1.44	TRAF family member-associated NFKB activator	NM_004180.2
SUSD2	1.57	−1.41	Sushi domain containing 2	NM_019601.3
Downregulated in FPGS Overexpression and Upregulated in FPGS Inhibition				
ALDH1A3[a]	−1.61[b]	1.85	Aldehyde dehydrogenase 1 family, member A3	NM_000693.2
GPR110	−1.58	1.70	G protein-coupled receptor 110	NM_153840.2
IGFBP6	−1.59	1.64	Insulin-like growth factor binding protein 6	NM_002178.2
ALDH3A2	−1.34	1.49	Aldehyde dehydrogenase 3 family, member A2	NM_001031806.1
DLEU2L	−1.31	1.49	Deleted in lymphocytic leukemia 2 like	NR_002771.1
FBXO6	−1.42	1.48	F-box protein 6	NM_018438.4
PPFIBP1	−1.64	1.43	PTPRF interacting protein, binding protein 1 (liprin beta 1)	NM_003622.2
RPL34	−2.46	1.43	Ribosomal protein L34	NM_000995.2
CDC25C	−1.55	1.39	Cell division cycle 25 homolog C (*Schizosaccharomyces pombe*)	NM_001790.3
RDM1	−1.42	1.37	RAD52 motif 1	NM_001034836.1

[a] A given gene is represented in the microarray set with multiple identifiers.
[b] Expression of gene might have been influenced by promoter DNA methylation changes.

changes in activities of other genes whose expression was directly affected by promoter CpG DNA methylation have likely contributed to the observed gene expression changes.

We identified several genes whose expression might be specifically influenced by FPGS modulation as suggested by the opposite direction of gene expression changes between FPGS overexpression and inhibition. Among these genes, we identified few genes, expression of which was likely regulated by promoter CpG DNA methylation changes in an inverse manner. In HCT116 cells, *ALDH1A3* was such a gene. The expression of this gene was also shown to be inversely regulated by promoter CpG DNA methylation changes in response to GGH modulation in HCT116 cells [12]. *ALDH1A3* encodes aldehyde dehydrogenase 1 family, member A3, which may be involved in the detoxification of aldehydes generated by alcohol metabolism and lipid peroxidation and in the oxidation of retinal to retinoic acid [23]. *ALDH1A3* overexpression is associated with a more aggressive cancer phenotype [24] and resistance of cancer cells to chemotherapy [25]. In MDA-MB-435 cells, we identified several such genes including *HLA-DPA1*, *THBS2*, *C1S* and *PLSCR1*. *HLA-DPA1* (major histocompatibility complex, class II, DP alpha 1) is expressed on antigen presenting cells

Table 3
List of the top genes associated with the FPGS-specific altered expression in the FPGS-modulated MDA-MB-435 breast cancer cells.

Gene Symbol	Fold Change (vs. Control)		Description	Accession
	FPGS Overexpression	FPGS Inhibition		
Upregulated in FPGS Overexpression and Downregulated in FPGS Inhibition				
HLA-DQA1	9.13	−5.75	PREDICTED: major histocompatibility complex, class II, DQ alpha 1, transcript variant 10	XM_936128.2
THBS2	2.13	−5.25[b]	Thrombospondin 2	NM_003247.2
C21orf34[a]	8.79	−4.48	Chromosome 21 open reading frame 34	NM_001005734.1
HLA-DOA	4.12	−3.95	Major histocompatibility complex, class II, DO alpha	NM_002119.3
C1S	1.59	−3.26[b]	Complement component 1, s subcomponent	NM_001734.2
HLA-DMB	3.19	−3.17	Major histocompatibility complex, class II, DM beta	NM_002118.3
CTHRC1[a]	4.19	−2.88	Collagen triple helix repeat containing 1	NM_138455.2
HLA-DPA1	2.82[b]	−2.87[b]	Major histocompatibility complex, class II, DP alpha 1	NM_033554.2
HLA-DRB4	4.04	−2.82	Major histocompatibility complex, class II, DR beta 4	NM_021983.4
HLA-DPB1	2.31	−2.76	Major histocompatibility complex, class II, DP beta 1	NM_002121.4
Downregulated in FPGS Overexpression and Upregulated in FPGS Inhibition				
BCHE[a]	−11.02	7.02	Butyrylcholinesterase	NM_000055.1
TRIM48	−11.38	3.67	Tripartite motif containing 48	NM_024114.2
ANKS1A	−1.92	2.83	Ankyrin repeat and sterile alpha motif domain containing 1A	NM_015245.2
SLITRK4	−1.80	2.60	SLIT and NTRK-like family, member 4	NM_173078.2
ST3GAL5[a]	−2.14	2.20	ST3 beta-galactoside alpha-2,3-sialyltransferase 5	NM_001042437.1
AKR1B1	−1.50	2.04	Aldo-keto reductase family 1, member B1 (aldose reductase)	NM_001628.2
DYNC1I1	−5.14	1.99	Dynein, cytoplasmic 1, intermediate chain 1	NM_004411.3
PLSCR1	−2.62[b]	1.97	Phospholipid scramblase 1	NM_021105.1
MIR1974	−1.65	1.97	microRNA 1974	NR_031738.1
PPARGC1A	−4.23	1.95	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	NM_013261.3

[a] A given gene is represented in the microarray set with multiple identifiers.
[b] Expression of gene might have been influenced by promoter DNA methylation changes.

and plays an important role in autoimmune diseases and parasitic infections [26,27]. *THBS2* encodes thrombospondin 2 that mediates cell-to-cell and cell-to-matrix interactions and functions as a potent inhibitor of tumor growth and angiogenesis [28,29]. *THBS2* downregulation by aberrant DNA methylation may be involved in angiogenesis of malignant ovarian tumors [30]. *C1S* encodes a serine protease, a major constituent of the human complement subcomponent C1 [31]. *PLSCR1* encodes phospholipid scramblase 1 involved in cell signaling, maturation and apoptosis and contributes to cancer development and responses to chemotherapeutic agents [32,33].

FPGS-induced polyglutamylation is an important determinant of the sensitivity of cancer cells to antifolates and 5-fluorouracil (5FU) [3,13,14]. Generally, FPGS overexpression increases, while FPGS inhibition decreases, chemosensitivity of cancer cells to antifolates including methotrexate and 5FU [34–38]. Using the same *in vitro* model of FPGS overexpression and inhibition utilized in the present study, we have previously demonstrated that FPGS overexpression enhances, while FPGS inhibition decreases, chemosensitivity of HCT116 and MDA-MB-435 cells to 5FU [13,14], the critical component of colorectal and breast cancer chemotherapy [39]. In the present study, we have observed several genes, expression change of which might have in part contributed to the FPGS modulation-induced changes in 5FU chemosensitivity [13,14]. In HCT116 and MDA-MB-435 cells, we observed downregulation of *TYMS* and *TK1* in response to FPGS overexpression, which may increase the sensitivity of these cells to 5FU. We also observed upregulation of *TK1* in the FPGS-inhibited MDA-MB-435 cells, which may decrease the sensitivity of these cells to 5FU. *TYMS* encodes thymidylate synthase whose expression and activity are well-established inverse determinants of 5FU efficacy [40,41]. *TK1* encodes thymidine kinase 1, high levels of which are associated with 5FU resistance [42–45]. We also observed downregulation of *ABCC5* and *SLC29A1* in response to FPGS overexpression in HCT116 and MDA-MB-435 cells. *ABCC5* encodes multidrug-resistance-associated protein 5 (MRP5) that facilitates the efflux of 5FU metabolites; MRP5 upregulation is associated with 5FU resistance, whereas MRP5 downregulation is associated with enhanced 5FU efficacy in cancer cells [46]. Therefore, the observed downregulation of *ABCC5* in response to FPGS overexpression would lead to the enhanced efficacy of 5FU. *SLC29A1* encodes equilibrative nucleoside transporter 1 (ENT1); high mRNA expression of ENT1 is associated with a poor response of colon cancer cells to 5FU [47,48]. Our data provide evidence that FPGS modulation significantly influences expression of genes involved in important biological pathways that might account for the observed effects of FPGS modulation on cancer treatment response [13,14]. Furthermore, our data provide a framework for future studies aimed at interrogating specific biological pathways associated with FPGS modulation and at exploring upstream and downstream targets of FPGS modulation.

The role of FPGS in human health and disease has not yet been clearly demonstrated. Our data indicate that FPGS modulation associated with significant changes in intracellular folate concentrations and folylpolyglutamate distribution can lead to altered global and promoter CpG DNA methylation and expression of genes involved in important biological pathways that might contribute to the development and progression of diseases that are linked to aberrant intracellular folate and one-carbon metabolism. Some of the observed altered gene expression appeared to be regulated by promoter CpG DNA methylation. We identified several differentially expressed genes involved in folate biosynthesis and one-carbon metabolism, which might in part have contributed to the observed change in 5FU chemosensitivity in response to FPGS modulation [13,14]. The potential role of FPGS modulation in DNA methylation and its associated downstream functional effects as well as in cancer treatment warrants further studies.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2015.10.019.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

Funding was provided by the Canadian Institutes of Health Research (Grant Number 14126 to YIK).

References

[1] Kim YI. Folate and colorectal cancer: an evidence-based critical review. Mol Nutr Food Res 2007;51:267–92.
[2] Shane B. Folate chemistry and metabolism. In: Bailey LB, editor. Folate in health and disease. Boca Raton, FL: CRC Press; 2010. p. 1–24.
[3] Moran RG. Roles of folylpoly-gamma-glutamate synthetase in therapeutics with tetrahydrofolate antimetabolites: an overview. Semin Oncol 1999;26:24–32.
[4] Lawrence SA, Titus SA, Ferguson J, Heineman AL, Taylor SM, Moran RG. Mammalian mitochondrial and cytosolic folylpolyglutamate synthetase maintain the subcellular compartmentalization of folates. J Biol Chem 2014;289:29386–96.
[5] Kim YI. Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility. J Nutr 2005;135:2703–9.
[6] Ly A, Hoyt L, Crowell J, Kim YI. Folate and DNA methylation. Antioxid Redox Signal 2012;17:302–26.
[7] Kulis M, Esteller M. DNA methylation and cancer. Adv Genet 2010;70:27–56.
[8] Li E, Jaenisch R. DNA methylation and methyltransferases. In: Natick EM, editor. DNA alterations in cancer: genetic and epigenetic changes. MA: Eaton Publishing; 2000. p. 351–65.
[9] Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042–54.
[10] McGuire JJ, Bertino JR. Enzymatic synthesis and function of folylpolyglutamates. Mol Cell Biochem 1981;38 Spec No:19–48.
[11] Zhou HR, Zhang FF, Ma ZY, Huang HW, Jiang L, Cai T, et al. Folate polyglutamylation is involved in chromatin silencing by maintaining global DNA methylation and histone H3K9 dimethylation in *Arabidopsis*. Plant Cell 2013;25:2545–59.
[12] Kim SE, Hinoue T, Kim MS, Sohn KJ, Cho RC, Cole PD, et al. gamma-Glutamyl hydrolase modulation significantly influences global and gene-specific DNA methylation and gene expression in human colon and breast cancer cells. Genes Nutr 2015;10:444.
[13] Cho RC, Cole PD, Sohn KJ, Gaisano G, Croxford R, Kamen BA, et al. Effects of folate and folylpolyglutamyl synthase modulation on chemosensitivity of breast cancer cells. Mol Cancer Ther 2007;6:2909–20.
[14] Sohn KJ, Smirnakis F, Moskovitz DN, Novakovic P, Yates Z, Lucock M, et al. Effects of folylpolyglutamate synthetase modulation on chemosensitivity of colon cancer cells to 5-fluorouracil and methotrexate. Gut 2004;53:1825–31.
[15] Laird PW, Zijderveld A, Linders K, Rudnicki MA, Jaenisch R, Berns A. Simplified mammalian DNA isolation procedure. Nucleic Acids Res 1991;19:4293.
[16] Bibikova M, Le J, Barnes B, Saedinia-Melnyk S, Zhou L, Shen R, et al. Genome-wide DNA methylation profiling using Infinium((R)) assay. Epigenomics 2009;1:177–200.
[17] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8.
[18] Shane B. Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism. Vitam Horm 1989;45:263–335.
[19] Yajnik CS, Deshpande SS, Jackson AA, Refsum H, Rao S, Fisher DJ, et al. Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study. Diabetologia 2008;51:29–38.
[20] Houshdaran S, Hawley S, Palmer C, Campan M, Olsen MN, Ventura AP, et al. DNA methylation profiles of ovarian epithelial carcinoma tumors and cell lines. PLoS One 2010;5:e9359.
[21] Pike BL, Greiner TC, Wang X, Weisenburger DD, Hsu YH, Renaud G, et al. DNA methylation profiles in diffuse large B-cell lymphoma and their relationship to gene expression status. Leukemia 2008;22:1035–43.
[22] Volkmar M, Dedeurwaerder S, Cunha DA, Ndlovu MN, Defrance M, Deplus R, et al. DNA methylation profiling identifies epigenetic dysregulation in pancreatic islets from type 2 diabetic patients. EMBO J 2012;31:1405–26.
[23] Sima A, Parisotto M, Mader S, Bhat PV. Kinetic characterization of recombinant mouse retinal dehydrogenase types 3 and 4 for retinal substrates. Biochim Biophys Acta 1790;2009:1660–4.
[24] Marcato P, Dean CA, Pan D, Araslanova R, Gillis M, Joshi M, et al. Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. Stem Cells 2011;29:32–45.
[25] Golubovskaya V, O'Brien S, Ho B, Heffler M, Conroy J, Hu Q, et al. Down-regulation of ALDH1A3, CD44 or MDR1 sensitizes resistant cancer cells to FAK autophosphorylation inhibitor Y15. J Cancer Res Clin Oncol 2015;141:1613–31.
[26] Lantermann A, Hampe J, Kim WH, Winter TA, Kidd M, Nagy M, et al. Investigation of HLA-DPA1 genotypes as predictors of inflammatory bowel disease in the German, South African, and South Korean populations. Int J Color Dis 2002;17:238–44.
[27] Zhang JH, Liu WQ, Li YL, Long XC. Studies on the association of human leukocyte antigen class II alleles with advanced hepatosplenic *Schistosomiasis japonica*. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 2005;23:6–9.

[28] Koch M, Hussein F, Woeste A, Grundker C, Frontzek K, Emons G, et al. CD36-mediated activation of endothelial cell apoptosis by an N-terminal recombinant fragment of thrombospondin-2 inhibits breast cancer growth and metastasis *in vivo*. Breast Cancer Res Treat 2011;128:337–46.
[29] Streit M, Stephen AE, Hawighorst T, Matsuda K, Lange-Asschenfeldt B, Brown LF, et al. Systemic inhibition of tumor growth and angiogenesis by thrombospondin-2 using cell-based antiangiogenic gene therapy. Cancer Res 2002;62:2004–12.
[30] Whitcomb BP, Mutch DG, Herzog TJ, Rader JS, Gibb RK, Goodfellow PJ. Frequent HOXA11 and THBS2 promoter methylation, and a methylator phenotype in endometrial adenocarcinoma. Clin Cancer Res 2003;9:2277–87.
[31] Gal P, Ambrus G, Zavodszky P. C1s, the protease messenger of C1. Structure, function and physiological significance. Immunobiology 2002;205:383–94.
[32] Huang Y, Zhao Q, Zhou CX, Gu ZM, Li D, Xu HZ, et al. Antileukemic roles of human phospholipid scramblase 1 gene, evidence from inducible PLSCR1-expressing leukemic cells. Oncogene 2006;25:6618–27.
[33] Fan CW, Chen CY, Chen KT, Shen CR, Kuo YB, Chen YS, et al. Blockade of phospholipid scramblase 1 with its N-terminal domain antibody reduces tumorigenesis of colorectal carcinomas *in vitro* and *in vivo*. J Transl Med 2012; 10:254.
[34] Kim JS, Lowe KE, Shane B. Regulation of folate and one-carbon metabolism in mammalian cells. IV. Role of folylpoly-gamma-glutamate synthetase in methotrexate metabolism and cytotoxicity. J Biol Chem 1993;268:21680–5.
[35] Aghi M, Kramm CM, Breakefield XO. Folylpolyglutamyl synthetase gene transfer and glioma antifolate sensitivity in culture and *in vivo*. J Natl Cancer Inst 1999;91: 1233–41.
[36] Romanini A, Lin JT, Niedzwiecki D, Bunni M, Priest DG, Bertino JR. Role of folylpolyglutamates in biochemical modulation of fluoropyrimidines by leucovorin. Cancer Res 1991;51:789–93.
[37] Wang FS, Aschele C, Sobrero A, Chang YM, Bertino JR. Decreased folylpolyglutamate synthetase expression: a novel mechanism of fluorouracil resistance. Cancer Res 1993;53:3677–80.
[38] Cheradame S, Etienne MC, Chazal M, Guillot T, Fischel JL, Formento P, et al. Relevance of tumoral folylpolyglutamate synthetase and reduced folates for optimal 5-fluorouracil efficacy: experimental data. Eur J Cancer 1997;33:950–9.
[39] Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 2003;3:330–8.
[40] Oguri T, Achiwa H, Bessho Y, Muramatsu H, Maeda H, Niimi T, et al. The role of thymidylate synthase and dihydropyrimidine dehydrogenase in resistance to 5-fluorouracil in human lung cancer cells. Lung Cancer 2005;49:345–51.
[41] Yoshinare K, Kubota T, Watanabe M, Wada N, Nishibori H, Hasegawa H, et al. Gene expression in colorectal cancer and *in vitro* chemosensitivity to 5-fluorouracil: a study of 88 surgical specimens. Cancer Sci 2003;94:633–8.
[42] van der Wilt CL, Backus HH, Smid K, Comijn L, Veerman G, Wouters D, et al. Modulation of both endogenous folates and thymidine enhance the therapeutic efficacy of thymidylate synthase inhibitors. Cancer Res 2001;61:3675–81.
[43] Wang W, Cassidy J, O'Brien V, Ryan KM, Collie-Duguid E. Mechanistic and predictive profiling of 5-fluorouracil resistance in human cancer cells. Cancer Res 2004;64:8167–76.
[44] Look KY, Moore DH, Sutton GP, Prajda N, Abonyi M, Weber G. Increased thymidine kinase and thymidylate synthase activities in human epithelial ovarian carcinoma. Anticancer Res 1997;17:2353–6.
[45] Fanciullino R, Evrard A, Cuq P, Giacometti S, Peillard L, Mercier C, et al. Genetic and biochemical modulation of 5-fluorouracil through the overexpression of thymidine kinase: an *in-vitro* study. Anti-Cancer Drugs 2006;17:463–70.
[46] Hagmann W, Jesnowski R, Faissner R, Guo C, Lohr JM. ATP-binding cassette C transporters in human pancreatic carcinoma cell lines. Upregulation in 5-fluorouracil-resistant cells. Pancreatology 2009;9:136–44.
[47] Kubota T. Real-time RT-PCR (TaqMan) of tumor mRNA to predict sensitivity of specimens to 5-fluorouracil. Mex Farm 2005;111:257–65.
[48] Tsujie M, Nakamori S, Nakahira S, Takahashi Y, Hayashi N, Okami J, et al. Human equilibrative nucleoside transporter 1, as a predictor of 5-fluorouracil resistance in human pancreatic cancer. Anticancer Res 2007;27:2241–9.