Effects of folate deficiency on gene expression in the apoptosis and cancer pathways in colon cancer cells

Petar Novakovic¹, Joanne M.Stempak¹,², Kyoung-Jin Sohn¹,³ and Young-In Kim¹,²,³,⁴

¹Institute of Medical Science, ²Department of Nutritional Sciences and ³Department of Medicine, University of Toronto, Toronto, Ontario, Canada M5S 1A8 and ⁴Division of Gastroenterology, St Michael’s Hospital, Toronto, Ontario, Canada M5B 1W8

To whom correspondence and requests for reprints should be addressed at: Room 7258, Medical Sciences Building, University of Toronto, 1 King’s Circle, Toronto, Ontario, Canada M5S 1A8. Tel: +416 978 1183; Fax: +416 978 8765; Email: youngin.kim@utoronto.ca

Folate is a B vitamin, deficiency of which appears to increase the risk of developing several malignancies including colorectal cancer. In contrast to the cancer-promoting effect of folate deficiency in normal tissues, several lines of evidence indicate that folate depletion suppresses the progression of existing neoplasms and enhances the sensitivity of cancer cells to chemotherapy. Folate mediates the transfer of one-carbon necessary for the de novo biosynthesis of purines and thymidylate, and hence is an essential factor for DNA synthesis and repair, and the maintenance of DNA integrity and stability. Folate deficiency induces DNA strand breaks, increases uracil misincorporation into DNA, impairs DNA repair and appears to induce apoptosis. Although the effects of folate depletion on DNA integrity and apoptosis and on subsequent cancer development, progression and treatment in colonic epithelial cells have been well characterized, it is largely unknown at present how folate depletion modulates specific upstream genes in apoptosis and cancer pathways that regulate these processes. We therefore investigated the effects of folate depletion on expression of genes involved in apoptosis and cancer pathways in four human colon adenocarcinoma cell lines in an in vitro model of folate deficiency. Apoptosis and cancer pathway-specific minigene microarray were used to screen for differentially expressed genes in response to folate deficiency, and the expression of seven most notably and consistently affected genes was confirmed by real time RT–PCR. Our data suggest that folate deficiency affects the expression of key genes that are related to cell cycle control, DNA repair, apoptosis and angiogenesis in a cell-specific manner. Cell-specificity in gene expression changes in response to folate deficiency is likely due to significant differences in molecular and phenotypic characteristics, growth rates and intracellular folate concentrations among the four cell lines.

Introduction

Folate is a water-soluble B vitamin that mediates the transfer of one-carbon necessary for the de novo biosynthesis of purines and thymidylate, and hence is an essential factor for DNA synthesis, replication and repair (1). Folate deficiency is associated with DNA strand breaks, chromosomal gaps and breakage, increased uracil misincorporation into DNA, impaired DNA repair and increased mutations in in vivo and in vitro models, thereby supporting a role for folate in the maintenance of DNA integrity and stability (2–8). DNA strand breaks and nucleotide deprivation are potent stimuli that activate the apoptotic pathway (9). Folate depletion in conjunction with nucleotide precursors (thymidine and hypoxanthine) deprivation has been shown to induce apoptosis in human HepG2 hepatoma (10), Chinese hamster ovary cells (11) and murine proerythroblasts (12).

Folate deficiency has been implicated in the development of several malignancies with the most supportive evidence existing for colorectal cancer (13). Although the mechanisms of the folate deficiency-mediated colorectal carcinogenesis still remain to be clearly elucidated, aberrancies of DNA synthesis, stability and integrity, and repair in the colorectum resulting from folate deficiency-induced disrupted one-carbon transfer reactions have emerged as a primary mechanism (14,15). In contrast to the cancer-promoting effect of folate deficiency in normal tissues, several lines of evidence indicate that intracellular folate depletion suppresses the progression of existing neoplasms and enhance the sensitivity of cancer cells to chemotherapy (16). In neoplastic cells, in which DNA replication and cell division occur at an accelerated rate, folate depletion or interruption of folate metabolism causes ineffective DNA synthesis, resulting in inhibition of tumor growth (16). This has been the basis for cancer chemotherapy using antifolate agents (e.g. methotrexate) and 5-fluorouracil (16). Folate deficiency has been shown to induce regression and suppress the progression of preexisting neoplasms in experimental models (17,18). Furthermore, folate deficiency has been shown to act synergistically with alkylating agents to increase DNA strand breaks and mutations, thereby augmenting their cytotoxic effect (19–23). Folate deficiency therefore appears to have dual effects on cancer development, progression and treatment depending on the stage of transformation of the target tissue (13). Folate deficiency has an inhibitory effect, whereas folate supplementation has a promoting effect on the progression of established neoplasms. In contrast, folate deficiency in normal tissues appears to predispose them to neoplastic transformation, and modest levels of folate supplementation suppress the development of tumor in normal tissues.

Although the effects of folate depletion on DNA integrity and apoptosis and on subsequent cancer development, progression and treatment in colonic epithelial cells have been well characterized, it is largely unknown at present how folate...
depletion modulates specific upstream genes in apoptosis and cancer pathways that regulate these processes. We therefore investigated the effects of folate depletion on expression of genes involved in apoptosis and cancer pathways using four human colon adenocarcinoma cell lines.

Materials and methods

Cell lines and culture

Four human colon adenocarcinoma cell lines, HCT116, Caco-2, HT29 and LS513, with different molecular and phenotypic characteristics (Appendix 1) were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in standard RPMI 1640 medium (Invitrogen, Gaithersburg, MD) containing 2.3 μM folic acid (control) or in custom RPMI 1640 medium (Invitrogen) free of folic acid (deficient). Growth medium was supplemented with dialyzed 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin-streptomycin and 0.1% fungizone. The folate-deficient medium therefore contained only folate acid present in dialyzed serum (0.6 nM). This level of folate in medium has previously been shown to physiologically sufficient to sustain growth of both untransformed and transformed mammalian cell lines in culture (24). Cells were maintained at 37°C in 95% humidity and 5% CO2 and passed every 4 days. Growth rates were determined by cell counts. Cells were harvested after 20 days of growth.

Intracellular folate assay

Intracellular folate concentrations were determined by a standard microtiter plate assay using Lactobacillus casei after cellular folate extraction and subsequent treatment with chicken pancreas conjugase as described previously (25). All analyses were performed in triplicate and repeated using three different cell lysates.

Deoxyuridine suppression test

Deoxyuridine suppression test was used to verify that intracellular folate depletion was functionally significant as described previously (24). Deoxyuridine suppression test assesses the de novo synthesis of thymidine on the basis of the competition between two pathways: the salvage pathway and the de novo pathway (26). The salvage pathway consists of phosphorylation of thymidine by thymidine kinase. The de novo pathway generates thymidate by methylating deoxyuridine monophosphate. Because the enzyme for the latter reaction, thymidylate synthase, requires methylenetetrahydrofolate as a substrate, this reaction is suppressed by exogenous deoxyuridine, whereas in folate-deficient cells, the [3H]thymidine incorporation without deoxyuridine.

Real time quantitative RT-PCR

Real time quantitative RT-PCR was used to confirm the data obtained from the microarray gene expression analysis as described previously (27). cDNA was generated from 5 μg of total RNA using random primers and the SuperScript III RNase H-Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. Real time hot-start PCR was performed at 95°C for 10 min in a 10 μl reaction volume containing 0.5 μM of each primer, 4 mM MgCl2 and 1 μl of cDNA template using the LC FastStart DNA Master SYBR Green 1 Kit in the LightCycler rapid thermal cycler system (Roche Diagnostics, Laval Quebec, Canada). Following hot-start PCR, 45 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 5 s and extension at 72°C for 10 s were performed. After amplification, the reaction mixture was cooled to 65°C for 15 s, and then by slowly raising the temperature to 95°C at 0.1°C in order to generate dissociation curve for each gene to ensure that non-specific amplification did not occur. All PCR products were analyzed by gel electrophoresis to ensure that the amplification was of anticipated length. Primers were designed using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA). primer–primer interactions were minimized by analysis using oligo 4.0 software (Molecular Biology Insights, Cascade, CO), and primers were synthesized by the ACGT (Toronto, Ontario, Canada). Primer sets were as follows: ATM (Sense: 5'-GAGAAATGCTCCATATGCTGAGA-3'; Antisense: 5'-AGGCTTGTGTTTTGCGATAGAG-3'), BAX (Sense: 5'-TGGAGGTCGACAGGATGATGTTG-3'; Antisense: 5'-TGCCACTGCGGAAAGACCT-3'), CTNNB1 (Sense: 5'-GCATTAGCTGATTGAATACT-3'; Antisense: 5'-GGATCTGCTCTCAG-3'), MDM-2 (Sense: 5'-ATGATTGAGAGGATGATTG-3'; Antisense: 5'-TCCGAAAGTCTGTTCAGG-3'), p21 (Sense: 5'-GGGACACAGCATGACATC-3'; Antisense: 5'-CCGTCTCAGTACAGGCT-3'), p53 (Sense: 5'-GTTGGCCTTGTTCGTTG-3'; Antisense: 5'-TCCCTCTTTTCTGGACAGATG-3'), VEGF (Sense: 5'-AGGGCAATTTCACTCAAGATTG-3'; Antisense: 5'-GGGCCACAGGATGCTTGTA-3'), and ALAS1 (Sense: 5'-AACGCCGCGTGTCGCTGTTG-3'; Antisense: 5'-CGAATCCTTGCGACATG-3').

Standard curves were prepared for all target genes and the endogenous reference gene, human 5-aminolevulinate dehydrate synthase 1 (ALAS1). Relative quantification was performed using LC Relative Quantification Software version 1.0 (Roche Diagnostics). All PCRs were performed in triplicate, and expression times were repeated with the same RNA for three different cultures. Interassay coefficients of variations ranged from 0.7 to 3.8% for ATM, 0.4 to 2.9% for BAX (BCL-2 associated X protein), 0.3 to 7.6% for CTNNB1 (cadherin-associated protein-p25 catenin), 0.2 to 5.2% for MDM-2 (p53 binding protein), 0.6 to 6.6% for p21, 0.5 to 9.9% for p53 and 0.4 to 10.5% for VEGF (vascular endothelial growth factor) among the four cell lines tested.

Western blot analysis

Total cellular lysates were obtained by incubating cells in RIPA solution containing protease inhibitors and ethylene glycol bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA) at 0.1 mg/ml (aprotinin at 2 mg/ml) (Roche Diagnostics). Supernatants were collected after centrifugation at 18 000 g for 30 min 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 15% 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 h at room temperature. To detect p53 and p21 protein expression, the membranes were incubated with anti-p53(DO-1) and p21 monoclonal antibody (Oncogene Research Products, Boston, MA) at a dilution of 1:3000, respectively, in PBS containing 5% skim milk for 16 h at 4°C. The p53 and p21 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 α-actin antibody (Sigma Aldrich Canada, Oakville, Ontario, Canada) at a dilution of 1:3000. All western analyses were repeated using three different cell lysates.

Statistical analysis

Comparisons of means between the control and folate-deficient groups were determined using Student’s t-test. Gene expression differences between the control and folate-deficient groups were determined using Student’s t-test. For all analyses, results were considered statistically significant if two tailed P-values were <0.05. Genes with a 1.5-fold or greater change in expression were reported. It is customary to set a higher cut-off in order to select genes with the most drastically changed expression. In the present study, the magnitude of change in gene expression in response to folate deficiency was modest with the majority of genes showing ~2-fold change on average and no genes showing >4-fold change. Therefore, we lowered the cut-off to
1.5-fold in order to avoid excluding any potential genes with modest changes in gene expression in response to folate deficiency. Analyses were performed using SPSS, version 4.1 (SPSS, Chicago, IL).

Results

Effect of folate deficiency on growth rate and intracellular folate concentrations

Compared with corresponding cells cultured in folate-sufficient medium, the folate-deficient cells demonstrated significant progressive retarded growth (Figure 1). The growth rates of the folate-sufficient and deficient cells were markedly different among the four cell lines (Figure 1).

Intracellular folate concentrations of cells cultured in folate-deficient medium were significantly lower (by 88–98%) than those of the corresponding cells cultured in folate-sufficient medium (P < 0.002, Figure 2). Intracellular folate concentrations of the folate-sufficient and deficient cells were markedly different among the four cell lines (Figure 2).

To determine whether the observed degree of intracellular folate depletion in the folate-deficient cells was functionally significant, the deoxyuridine suppression test was performed. The folate-deficient cells were significantly less suppressed by exogenous deoxyuridine, resulting in significantly higher [3H]thymidine incorporation into DNA, compared with the corresponding folate-sufficient cells (P < 0.01, Figure 3), suggesting significant functional intracellular folate depletion in the folate-deficient cells. This effect was abolished by pre-incubation with folinic acid (Figure 3).

Effect of folate deficiency on gene expression in apoptosis and cancer pathways

Apoptosis and cancer pathway-specific mini-microarrays were used to screen for differentially expressed genes in response to folate deficiency in HCT116 and Caco-2 cells. A total of 5 (2 up and 3 downregulated) and 12 (9 up and 3 downregulated) apoptosis genes were differentially expressed in the folate-deficient HCT116 and Caco-2 cells, respectively, compared with the corresponding folate-sufficient cells (Table I). Only one apoptosis-related gene was differentially expressed (in the same direction) in response to folate deficiency in both HCT116 and Caco-2 cells (Table I).

A total of 13 (11 up and 2 downregulated) and 23 (14 up and 9 downregulated) cancer pathway genes were differentially expressed in the folate-deficient HCT116 and Caco-2 cells, respectively, compared with the corresponding folate-sufficient cells (Table II). Six cancer pathway genes were differentially expressed in response to folate deficiency in both HCT116 and Caco-2 cells: four in the same direction and two in the opposite directions (Table II).

Most notably and consistently affected genes were ATM, BAX, cadherin-associated protein-β-catenin 1 (CTNNB1),
Fig. 2. Intracellular folate concentrations of HCT116 (A), Caco-2 (B), HT29 (C) and LS513 (D) cells cultured in control (F⁺) and folate-deficient (F⁻) RPMI 1640 medium. Intracellular folate concentrations were determined by a standard microtiter plate assay using Lactobacillus casei after cellular folate extraction and subsequent treatment with chicken pancreas conjugase. Values are expressed as the mean and standard deviation of triplicate measurements (‘P < 0.002).

Fig. 3. Deoxyuridine suppression test was used to verify that intracellular folate depletion was functionally significant in HCT116 (A), Caco-2 (B), HT29 (C) and LS513 (D) cells cultured in control (F⁺) and folate-deficient (F⁻) RPMI 1640 medium. In folate-replete cells, the incorporation of [³H]thymidine into DNA is suppressed by exogenous deoxyuridine, whereas in folate-deficient cells, the degree of suppression is less pronounced because of impaired de novo synthesis of thymidylate and greater use of the salvage pathway (i.e. higher [³H]thymidine incorporation). Preincubation of these cells with folinic acid (100 μM) resulted in a correction of the deoxyuridine suppression test in the folate-deficient cells whereas the values remained unchanged in the control group. This demonstrates that repletion of the cellular folate pool in the folate-deficient cells resulted in a normal synthesis of thymidylate through the de novo pathway and therefore indicates that the abnormal deoxyuridine suppression observed in the deficient cells was due to a cellular deficiency of folate. Values are expressed as the mean and standard deviation of triplicate measurements. (‘P < 0.01).
Gene expression analysis on the seven genes using real time RT-PCR in four cell lines is presented in Table III. The direction of change in gene expression in response to folate deficiency was consistent between superarray and real time RT-PCR analyses, although the magnitude of change was different. The direction of change in gene expression was consistent in at least three cell lines for the CTNNB1, MDM-2, CDKN1A, p53 and VEGF genes. However, in some cell lines, the magnitude of change was either non-significant or very modest, albeit significant, for some of these genes. Even if those results that are not significant were eliminated, the
Data were normalized to the housekeeping gene ALAS1.

*p ≥ 0.05.

![Western blot analysis to determine the effects of folate deficiency on p53 and p21 protein expression in HCT116 and Caco-2 cells.](image)

**Discussion**

Using the previously characterized in vitro model of folate deficiency established in our laboratory (24), we determined the effects of folate deficiency on expression of genes involved in apoptosis and cancer pathways in human colon adenocarcinoma cell lines. We deliberately chose a severe degree of folate deficiency to maximize a chance of observing any effect of folate deficiency. Colon adenocarcinoma cells cultured in folate-deficient medium were viable but exhibited significant progressive retarded growth. A functionally significant degree of folate depletion was induced in the folate-deficient cells, thereby providing an appropriate in vitro model to study the effect of folate deficiency on gene expression.

It is apparent from the apoptosis and cancer pathway-specific mini-microarray analysis that the effect of folate deficiency on gene expression is highly cell-specific, given the low concordance rate of differentially expressed genes between HCT116 and Caco-2 cell lines. Furthermore, the real time RT–PCR analysis has demonstrated that the direction and magnitude of change in expression of the seven genes selected for analysis in response to folate deficiency are not uniformly consistent across all four cell lines. However, in five of the seven genes, the change in gene expression was in the same direction in three of the four cell lines. Cell-specificity in gene expression changes in response to folate deficiency is not entirely surprising because of significant differences in molecular and phenotypic characteristics (Appendix 1), growth rates (Figure 1) and intracellular folate concentrations (Figure 2) among the four cell lines. Therefore, it appears to be more appropriate to determine changes in gene expression involving apoptosis and cancer pathways and analyze data in a cell-specific manner rather than drawing a generalized conclusion by taking all cell lines into consideration. Our data also suggest that changes in gene expression observed in a single cell line cannot be extrapolated to other cell lines.

The most significant effect of folate deficiency on gene expression was observed in HCT116 cells in which all seven genes selected for real time RT–PCR analysis demonstrated a statistically significant 1.5-fold or greater change in gene expression in response to folate deficiency. This suggests that HCT116 cells might be particularly susceptible to the effect of folate deficiency as related to apoptosis and cancer pathways. The most notable difference between the HCT116 and the other three colon cancer cell lines is that HCT116 cells exhibit microsatellite instability due to an inactivating mutation in a mismatch repair gene MLH1 (28) whereas the other three colon cancer cell lines are microsatellite stable. Whether impaired DNA mismatch repair has contributed to an increased susceptibility of HCT116 cells to the effect of folate deficiency on expression of these genes involved in apoptosis and cancer pathways is of great interest and warrants further investigation.

Drawing a generalized conclusion concerning the effect of folate deficiency on gene expression across all four cell lines may not be appropriate because of cell-specific gene regulation in response to folate deficiency as discussed previously. However, a careful analysis that eliminates the expression changes either statistically not significant or <1.5-fold suggests that folate deficiency upregulates ATM, BAX, CTNNB1,
Fig. 5. A hypothetical model of cellular responses to folate deficiency in HCT116 cells.

MDM-2 and CDKN1A and downregulates p53, and VEGF. The downregulatory effect of folate deficiency on p53 gene expression is a surprising finding because it might be expected that folate deficiency-induced DNA damage and instability should upregulate p53 in order to invoke either DNA repair or apoptosis (29). However, p53 downregulation in response to folate deficiency is consistent with previously reported findings. Studies from this laboratory have previously shown that severe folate deficiency decrease steady-state levels of p53 mRNA in the colonic mucosa of rats (6,30) due to p53 strand breaks. It appears that exons 5–8 of the p53 gene is particularly susceptible to DNA strand breaks in response to folate deficiency as opposed to other genes studied and p53 strand breaks occur before genomic DNA strand breaks (5,6). Crott et al. (31) have recently confirmed a decline in p53 transcript by a microarray analysis in the colonic mucosa of rats placed on a less severe degree of folate deficiency. VEGF downregulation in response to folate deficiency observed in the present study is also consistent with the findings from a previous study that demonstrated using a microarray analysis a significant decrease in VEGF expression in the colonic mucosa of rats placed on a moderate degree of folate deficiency (31). VEGF plays a critical role in angiogenesis, which is important for tumor invasion and metastasis (32). The downregulatory effect of folate deficiency on VEGF expression in colon cancer cells is of great interest because it would suggest that folate deficiency might play an inhibitory role in tumor invasion and metastasis and thus warrants further investigation. The present study is the first to demonstrate that folate deficiency decreases p53 and VEGF transcripts in human colonic epithelial cells.

Upregulation of ATM, CTNNB1, BAX and CDKN1A is consistent with compensatory cellular responses to folate deficiency-induced DNA damage and instability; in response to injury, cells attempt to either induce cell cycle arrest to facilitate DNA repair or move the cells to apoptosis in the event that DNA damage is more severe and non-reparable (33). ATM is a kinase that controls cell cycle checkpoint signaling pathways required for cell response to DNA damage and for genomic stability; it activates p53 via phosphorylation (34). β-catenin, the product of CTNNB1, increases the transcription of p10ARF, which inactivates MDM-2, thereby stabilizing the amount of p53 available to mediate its transcriptional output (35). CDKN1A encodes a cyclin-dependent kinase inhibitor, p21CIP1, which inhibits G and S phase progression, thereby allowing time for DNA repair in response to cell injury (36). The pro-apoptotic, BCL-2 repressor gene, BAX, promotes apoptosis (37). However, p53 downregulation is not consistent with this model because upregulation of ATM and CTNNB1 should have upregulated p53 while upregulation of CDKN1A and BAX should have been the result of p53 upregulation (33). Upregulation of MDM-2 is consistent with p53 downregulation. MDM-2 downregulates p53 transcription and binds to p53 protein, thereby decreasing its activity and accelerating its degradation (38). However, MDM-2 upregulation is not consistent with upregulation of ATM, CTNNB1, CDKN1A and BAX. These data suggest that cellular responses for cell cycle control, DNA repair and apoptosis in response to folate deficiency may be independent of the p53 pathway, which warrants further investigation.

We propose a model that may explain the seemingly paradoxical increase in ATM (2.3-fold), BAX (2-fold), CTNNB1 (2-fold), MDM-2 (3.5-fold) and CDKN1A (2.2-fold) expression, despite a decrease (1.5-fold) in p53 mRNA expression using our data obtained in HCT116 cells (Figure 5). Although p53 mRNA expression was decreased by 1.5-fold in response to folate deficiency, p53 protein expression did not change significantly and p53 activity might have remained stable or increased. Several potential mechanisms exist that may explain the stability of p53 protein levels, despite decreased p53 mRNA expression. Folate deficiency is known to cause DNA damage and strand breaks (2–8), which may explain the 2.3-fold increase in the expression of the stress kinase, ATM. ATM-mediated phosphorylation of p53 functions to stabilize p53 by interfering with MDM-2 association (34,39), whereas phosphorylation of MDM-2 by ATM functions to reduce the ability of MDM-2 to promote the nucleo–cytoplasmic shuttling of p53 and its subsequent degradation, thereby enabling p53 accumulation (40). Thus, the increase in expression of ATM observed in our study may be one mechanism by which p53 protein activity was potentially increased or stabilized despite the observed 1.5-fold decrease in p53 mRNA expression.
CTNNB1 mRNA expression was also observed to be increased by ~2-fold in response to folate deficiency. This increase in CTNNB1 expression may also function to stabilize p53 protein activity despite the decrease in p53 mRNA expression. β-catenin, the product of CTNNB1, has been shown to possess the ability to increase the amount of transcriptionally active p53, at least in part through its ability to induce the transcription of p19ARF [p14ARF in humans] (35). p19ARF functions to inactivate MDM-2 E3 ligase and in so doing acts to stabilize the amount of p53 available to mediate its transcriptional output (35). The HCT116 cell line is known to contain a point mutation in the K-ras oncogene (41). Mutant forms of the K-ras oncogene are known to result in elevated levels of E2F1 expression, thereby enabling an independent pathway for the upregulation of p19ARF, and thus subsequent inhibition of MDM-2 mediated p53 degradation as described above (42). Therefore, despite the observed 3.5-fold increase in MDM2 expression and 1.5-fold decrease in p53 mRNA expression, overall p53 protein activity may have remained stable or even increased as a result of the combined effects of increased ATM, β-catenin and perhaps K-ras. In fact, the observed increase in MDM-2 expression may be a consequence of the p53–MDM-2 auto-regulatory feedback loop. The increased p53 activity may have stimulated MDM-2 synthesis; however, as mentioned above, the p53 inhibition/degradation half of the loop was inhibited due to both ATM and β-catenin, thus preventing the completion of the cycle and perpetuating the synthesis of MDM-2. There are also several other post-translational mechanisms that activate p53 and prevent p53 degradation without changes in p53 mRNA levels (43). There is overwhelming evidence that suggest that the induction of p53 in response to DNA damaging agents is controlled at the post-transcriptional levels, primarily through enhanced stability of the protein (43). Therefore, it is feasible that despite a decrease in p53 mRNA expression and stable p53 protein expression, p53 activity might have remained unchanged or even increased.

In addition to the gene expression study conducted in the rat colon cited previously (31), two other studies have investigated the effect of folate deficiency on gene expression in human cell lines (44,45). Jhaiveri et al. (44) reported eight differentially expressed genes in response to folate depletion in human nasopharyngeal epidermoid carcinoma KB cells including downregulation of cell adhesion molecule, H-cadherin. Courtemanche et al. (45) found that folate deficiency activated base and nucleotide excision repair genes and repressed folate-related genes in primary human lymphocytes primarily because of folate deficiency-induced DNA strand breaks, apoptosis, cell cycle arrest and growth retardation. We limited the study to test the effects of folate deficiency on gene expression patterns in established human colorectal cancer cell lines and hence the findings cannot be extrapolated to normal human colonic epithelial cells or to primary cells isolated from human colorectal cancer. Given the potentially important role of folate in DNA synthesis, repair, stability and apoptosis, hence its role in cancer development and treatment, future studies are required to elucidate the effect of folate deficiency and supplementation on expression of genes involved in these critical pathways not only in human colon cancer cell lines but also in normal human colonic epithelial cells.

In summary, our data suggest that folate deficiency affects the expression of key genes that are related to cell cycle control, DNA repair, apoptosis and angiogenesis in human colon cancer cells in a cell-specific manner. Depletion intratumor folate levels and interruption of intratumor folate metabolism without jeopardizing folate status of normal tissues might be an attractive strategy to enhance the sensitivity of cancer cells to chemotherapeutic agents. Elucidating specific genes affected by folate depletion in cancer cells will allow for a better understanding of the mechanism of action associated with the folate depletion-enhanced cytotoxicity on cancer cells. Furthermore, it will allow for the more effective use of folate deprivation to potentiate the effect of cancer chemotherapeutic agents by utilizing a more gene-specific approach.

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Conflict of Interest Statement: None declared.

References

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Appendix 1 Phenotypic and molecular characteristics of four human colon cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HCT116</th>
<th>Caco-2</th>
<th>HT29</th>
<th>LS513</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology Growth properties</td>
<td>Epithelial-like Adherent</td>
<td>Epithelial-like Adherent; upon reaching confluence, the cells express characteristics of enterocyte differentiation</td>
<td>Epithelial-like Adherent</td>
<td>Epithelial-like Adherent</td>
</tr>
<tr>
<td>Chromosome no.</td>
<td>45 (sterile chromosome number is near diploid with the modal number at 45)</td>
<td>96 (sterile modal number)</td>
<td>71 (sterile modal number) is hypotriploid</td>
<td>Two sterile modal numbers: 51 and 52</td>
</tr>
<tr>
<td>Molecular features</td>
<td>Positive for TGFβ1 and TGFβ2 and positive for keratin</td>
<td>Positive for expression of retinoic acid binding protein I and II and expresses receptors for heat stable enterotoxin and epidermal growth factor</td>
<td>Positive for expression of c-myc, K-ras, H-est, N-ras, Myb, sis and fos oncogenes, N-myc oncogene not detected. Expresses receptors for urokinase, vitamin D and human adrenergic alpha2A- oncogenes: myc;+; ras++; myb++; fos++; src++; p53++; abl--; ras: src=</td>
<td>Expresses MHC class I antigens HLA and β-2 microglobulin. MHC class II antigens (HLS-DR, DQ and DP not detected. -Expresses CEA and ICAM-1. TGFβ1 is inhibitory for proliferation, whereas TGFβ2 has no effect on growth</td>
</tr>
<tr>
<td>Gene mutations</td>
<td>MLH1, MSH3 A8, MSH6 C8, K-Ras, p14ARF, p16, BAX G8, β-catenin, DCC, Axin2, CBF2, E2F4, GRK4, Helicase q1, MBD4 10A, RASSO 9A, RIZ 9A, TGFBRII</td>
<td>p53</td>
<td></td>
<td></td>
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