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Original Article

Effects of folic acid deficiency on genetic damage in colorectal cancer cells

Zhen Wang, Changlei Su

Department of Colorectal Surgery, The Second Affiliated Hospital of Harbin Medical University, Harbin 150086, Heilongjiang, China

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Abstract: Objective: To explore the effect of folic acid deficiency on genetic damage and mRNA expression in colorectal cancer cells. Methods: We cultured human colonic epithelial cells ccd-841-con and colonic adenocarcinoma cells Caco-2 with RPMI1640 medium in folic acid-deficient concentration (22.6 nM) and standard concentration (2260 nM), respectively. Cytokinesis-block micronucleus cytometer was used to evaluate and compare the genetic damage of the tested cells. Poly(a) tailing method and dual luciferase reporter gene detection system were used to analyze the expression of miR-200a and its relationship with miR-190. Furthermore, the miR-190 expression was measured by RT-qPCR. Results: When folic acid was deficient for 21 days, the frequency of genetic damage was increased in both types of tested cells, and micronucleus, a marker associated with chromosome breakage, was dominant ($P < 0.01$). miR-200a targeted the 3'-UTR region of miR-190. In colonic epithelial cells ccd-841-con, the transcript levels of miR-200a and miR-190 were upregulated when folic acid was depleted for 21 days ($P < 0.01$). Conclusions: Folate deficiency can cause cytogenetic damage and affect the expression of miR-200a and miR-190 in rectal cancer cells.

Keywords: Folic acid deficiency, miR-200a, miR-190, gene expression, genetic damage

Introduction

Folic acid is abundantly present in green leafy vegetables, participates in the synthesis of purines and pyrimidines and various methylation reactions, and plays a key role in protein synthesis as well as cell division and growth. The one-carbon metabolism mediated by folic acid is crucial to the stability and function of the genome, and affects the occurrence and development of tumors [1]. Intersecting pathways and cycles of one-carbon metabolism rely on folate-activated one-carbon intermediates that are assigned to different pathways depending on the conditions, and disturbances in mechanisms affecting folate intermediates may lead to cancer development [2]. Folate deficiency increases uracil misincorporation, DNA strand breaks, chromosomal breaks, and malignant transformation. In addition to its impact on DNA damage, folate deficiency can also affect DNA repair [3]. Furthermore, folate deficiency has been associated with hypermethylation in specific gene regions, especially tumor suppressor genes [4]. Studies have shown that folic acid deficiency is related to the occurrence of various cancers such as colorectal cancer, ovarian cancer, breast cancer, and cervical cancer. Kim et al. [5] believed that a deficiency of folic acid could affect cell proliferation, leading to abnormalities in cell shape and physiology, and promoting the occurrence and progression of cancers. Conversely, folic acid supplementation may have an antitumor effect. It is suggested that folic acid and its one-carbon metabolism play an important role in the occurrence and progression of cancers.

Insufficient supply of folic acid in human body is a common worldwide phenomenon, and disturbance of folic acid absorption in intestine is often the main reason [6]. In 2004, it was found that the apparent absorption coefficient of folic acid was 0.65-0.97, with a mean value of 0.79, and the amount of folic acid absorbed from

Table 1. Main instruments and equipment

Name of experimental instrument	Model of experimental instrument	Manufacturers and brands of experimental instruments
Ultraviolet visible photometer	UV-1800	Shanghai mepec
Ultra low temperature freezer	MDF-192	Sanyo
Optical microscope	VANOX-S	Olympus, Japan
Barnstead	DZG-303A	Hongke
Microplate chemiluminescence meter	Varioskan LUX	Semefi
Gradient PCR instrument	VeritiTM 96	ABI
Micro spectrophotometer	Nano Photometer	IMPLEN
Constant temperature culture oscillator	ZHWY-211B	Shanghai Zhicheng
Overspeed freezing centrifuge	22R	BECKMAN
Manual sealing machine	SF-200	Shipley
Carbon dioxide incubator	MCO-15AC	Sanyo
Electric thermostatic water bath box	HH WZI	Beijing Changfeng
Vertical electrophoresis apparatus	VE-180	Shanghai Tianneng
Gel imager	BioDOC-1T2	UVP
Super clean workbench	SW-CJ-2FD	Suzhou Antai
Vertical pressure steam sterilizer	LDZX-50KBS	Shanghai Shen'an

food was 248-1762 nmol/d, with a mean value of 826 nmol/d [7].

As one of most active coenzymes in enzyme metabolism, folic acid is involved in the metabolic cycle of methionine. Folic acid is also involved in metabolism of epinephrine and choline. Through the actions of enzymes such as folate reductase and dihydrofolate reductase, the synthesis of organic substances such as acid and folic acid forms methyltetrahydrofolate, which serves as a carrier of one carbon unit. Methyltetrahydrofolate participates in the biosynthesis of crucial molecules including purines and pyrimidines, which are building blocks of DNA and RNA [8]. The role of folic acid is crucial in one-carbon metabolism. Normally, a one-carbon unit from serine or glycine is transferred to tetrahydrofolate to form 5,10-methylenetetrahydrofolate. 5,10-methylenetetrahydrofolate can be used in biosynthesis of thymidine deoxyribose, purines and methionine. After being oxidized to leucovorin, it can participate in the biosynthesis of purine, and after being reduced to 5-methyltetrahydrofolate, it can be involved in the biosynthesis of methionine [9]. Under action of ATP-dependent adenosyltransferase, adenosine is transferred to methionine to form S-adenosylmethionine (SAM). SAM is an extremely important molecule and has an unstable methyl group, which can be used for in vivo biosynthesis. Nearly 100 methylation reactions provide their methyl groups, including DNA, RNA and protein methylation [10]. It has been proven that miR-200a targets core MMR recognition complex MSH2-MSH6 in colon tumor cells. Overexpressed miR-200a can reduce MSH2 protein levels and alleviate G2/M induced by 5-memidine phase cell damage stagnated. Therefore, we hypothesized that miRNAs may respond to changes in folic acid supply conditions, regulate corresponding target genes and change related biological processes [11]. Based on this, our study focused on the specific miRNA expression changes caused by folate deficiency as well as the target gene miR-190 and its regulation.

Materials and methods

Experimental cell lines

Adherent epithelial cells including human colorectal adenocarcinoma cells Caco-2, human normal colon epithelial cells CCD-841-CoN and human embryonic kidney cells HEK293 used in this study were all purchased from the cell bank of Chinese Academy of Sciences.

Main equipment and instruments

The equipment and instruments used in this study are listed in **Table 1**.

Table 2. Main chemical reagents

	Name of reagent required for experiment	Brand
Reagents required for routine cell culture and intervention culture	1% L-Glutamine (cell culture grade)	GIBCO
	0.25% trypsin solution	GIBCO
	Dialysis (without folic acid) fetal bovine serum	GIBCO
	Calf Serum	GIBCO
	RPMI-1640 liquid medium (without folic acid)	GIBCO
	DMEM high glucose liquid complete medium	GIBCO
	0.1% penicillin-streptomycin (cell culture grade)	GIBCO
Reagents required for Western blot experiment	Skim milk powder	BIOSHARP
	PVDF membrane	MILLIPORE
	Cell Lysates	Solarbio
	PMSF	Solarbio
	BCA Protein Concentration Assay Kit	Blue sky
	TEMED	Solarbio
	Tween 20	BIOSHARP
	APS	SIGMA
	ECL Western Blot Luminescent Substrate	MILLIPORE
	Pre-stained color protein molecular weight marker maker	Blue sky
	Primary antibody to MSH2	ABCAM

Main chemical reagents

The main chemical reagents used in this study are listed in **Table 2**.

Routine cell culture

Human normal colon cells CCD-841-CoN and human colorectal adenocarcinoma cells Caco-2 were cultured in RPMI-1640 medium with 8% neonatal calf serum, 1% L-GLU and 0.1% penicillin/streptomycin. The human embryonic kidney cell line HEK293 was cultured in high glucose DMEM with 8% calf serum at the above concentration. This was used for targeting verification of miR-200a and miR-190.

Cell intervention culture

CCD-841-CoN and Caco-2 cells were intervened with modified RPMI1640 medium. The concentration of folic acid was set according to concentration of lymphocyte genome stability maintenance commonly used in our laboratory. The concentration of folic acid deficiency was 22.6 nM, and the sufficient concentration was 2260 nM [11]. Other components remained unchanged. A 25 cm cell culture flask was seeded with 5×10^5 cells/flask for static culture at 37°C in a CO_2 incubator. The medium was replaced every 2-3 days. After 21 days of intervention culture, an intervention model of long-term deficiency of folic acid in vitro was proposed.

miRNA test methods

The relative expression of miR-200a and miR-190 was analyzed by real-time fluorescent quantitative PCR technology. The total RNA was extracted and purified by Trizol method to ensure that the purity met the requirements. For PCR, agarose gel electrophoresis was used to detect PCR products. U6 was used as an external reference gene, and $2^{-\Delta\Delta Ct}$ was used to represent the relative expression level. Fluorescent quantitative PCR kits were provided by Treasure Bioengineering (Dalian) Co., Ltd., and all detection operations were carried out in strict accordance with the instructions. RNA was reversed to cDNA using the PrimeScript First Strand cDNA Synthesis Kit. Using cDNA as a template, real-time PCR amplification was carried out in ABI 7900 system using the following primers and SYBR Green Master Mix. The expression levels of miR-190 and miR-200a were analyzed by $2^{-\Delta\Delta Ct}$ method. Primer sequences: miR-190 sense strand: 5'-CCAAAT GCTACCATCCAGCC-3', antisense strand: 5'-TC-TGGCTTCCCTTAACCAAAA-3'; miR-200a sense strand: 5'-CACCGCCTCCCATTGTC-3', antisense

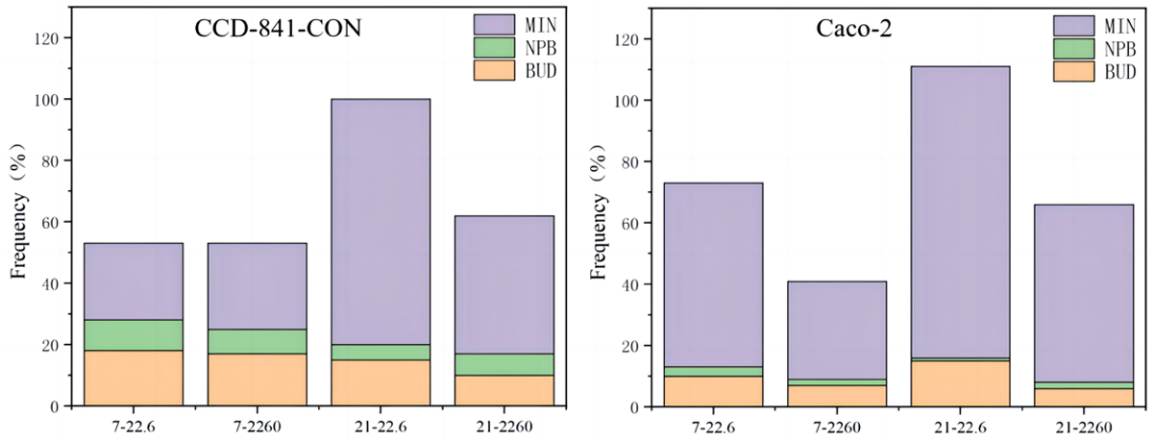


Figure 1. Effects of folic acid deficiency on genomic damage in CCD-841-CoN and Caco-2 cells.

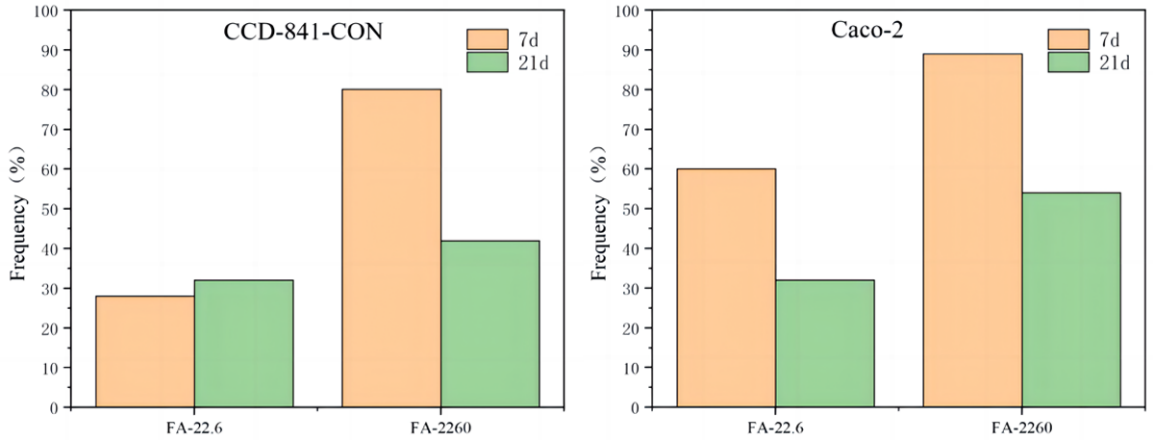


Figure 2. Effects of long-term folic acid deficiency on micronucleus of CCD-841-CoN and Caco-2 cells.

strand: 5'-CACAGGAAGTCAGTTCAGACC-3'; U6 sense strand: 5'-CTCGCTTCGGCAGCACA-3', antisense strand: 5'-AACGCTTCACGAATTTGC-GT-3'.

Statistical analysis

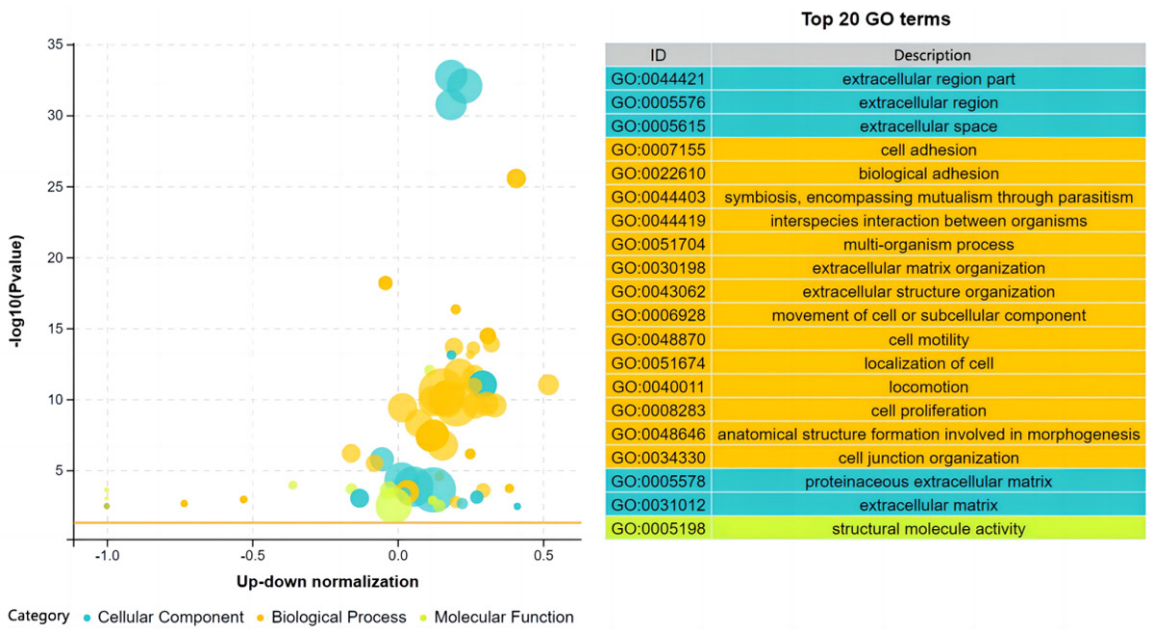
This study used SPSS 22.0 software for statistical analysis. The t test and one-way analysis of variance were carried out for data processing. $P < 0.05$ means the difference is statistically significant. Analysis of data among more than two groups was performed by one-way ANOVA with post hoc Bonferroni test.

Results

At 7 days, human normal colon epithelial cells CCD-841-CoN showed no genomic damage compared with normal controls, but at 21 days, folic acid deficiency increased gene damage. In human colorectal adenocarcinoma cells Caco-2, damage to genome increased gradually with long-term lack of folic acid. See **Figure 1**.

At 7 days, there was no difference in micronucleus (MN) between CCD-841-CoN and normal controls, but at 21 days, effect of folic acid deficiency on MN was increased. This phenomenon was more obvious in Caco-2 cells. The MN continued to increase with long-term lack of folic acid from day 7 to day 21 ($P < 0.01$). See **Figure 2**. It is indicated that when folic acid is continuously deficient, both normal cells and cancer cells can cause increase in gene instability such as DNA strands and chromosomal breaks.

In vertebrates, miR-190 gene was predicted to contain seed region of miR-200a by miRNA tar-



Top 20 GO terms

ID	Description
GO:0044421	extracellular region part
GO:0005576	extracellular region
GO:0005615	extracellular space
GO:0007155	cell adhesion
GO:0022610	biological adhesion
GO:0044403	symbiosis, encompassing mutualism through parasitism
GO:0044419	interspecies interaction between organisms
GO:0051704	multi-organism process
GO:0030198	extracellular matrix organization
GO:0043062	extracellular structure organization
GO:0006928	movement of cell or subcellular component
GO:0048870	cell motility
GO:0051674	localization of cell
GO:0040011	locomotion
GO:0008283	cell proliferation
GO:0048646	anatomical structure formation involved in morphogenesis
GO:0034330	cell junction organization
GO:0005578	proteinaceous extracellular matrix
GO:0031012	extracellular matrix
GO:0005198	structural molecule activity

Figure 3. Potential binding sites of miR-200a and miR-190 3'UTR.

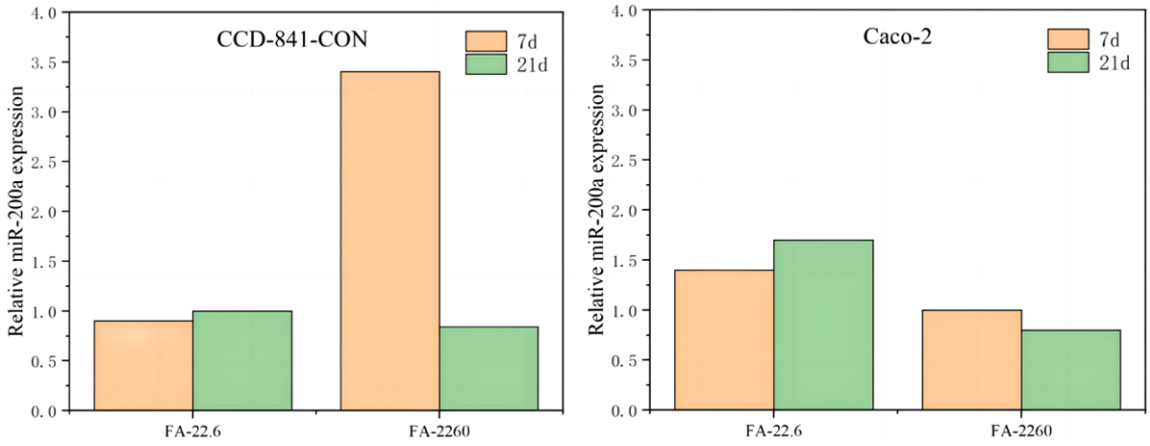


Figure 4. Changes of miR-200a in CCD-841-CoN and Caco-2 cells under folic acid sufficient/deficiency conditions. U6 was used as an internal reference.

get site prediction tool, and miR-200a may be an important potential target of miR-190 3'UTR. See **Figure 3**.

When folic acid was deficient for a long time, miR-200a was up-regulated in human normal colon epithelial cell CCD-841-CoN and colon cancer cell Caco-2. Compared with that in the controls, the miR-200a was up-regulated at 7 days and 21 days in human colorectal adenocarcinoma Caco-2 cells ($P < 0.01$). In normal colon epithelial cells, miR-200a was down-regulated at 7 days while up-regulated at 21 days. See **Figure 4**. The up-regulation of miR-200a often occurs in malignant tumors or precancerous lesions. It is indicated that long-term deficiency of folic acid in normal cells may lead to tumorigenesis of normal cells.

The mRNA levels of miR-190 showed differences between normal colon epithelial cells CCD-841-CoN and colorectal adenocarcinoma cells Caco-2. Furthermore, the expression of miR-200a was influenced by the deficiency/adequacy of folic acid, which regulated the mRNA levels of miR-190 gene. In Caco-2 cells, the mRNA

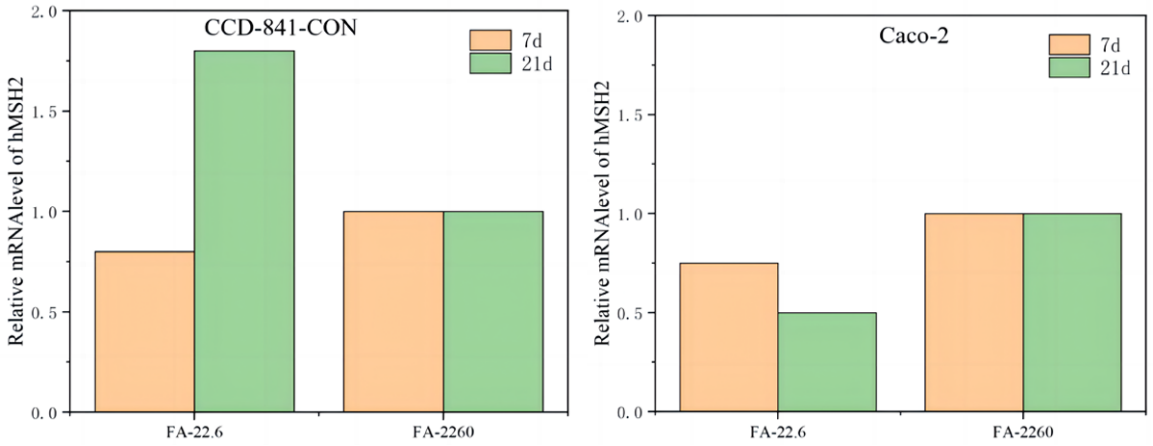


Figure 5. Changes of hMSH2 mRNA in CCD-841-CoN and Caco-2 cells under long-term folic acid deficiency. GAPDH served as an internal reference.

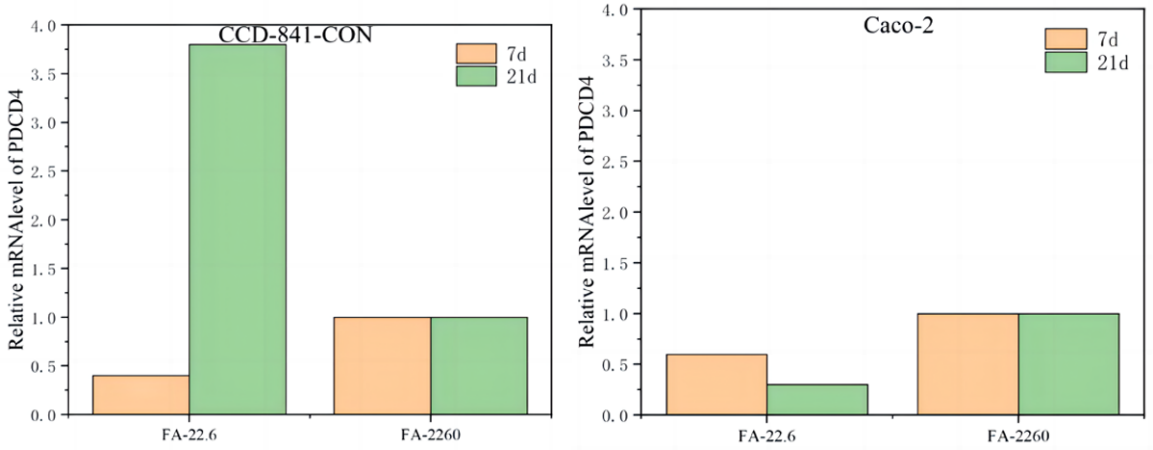


Figure 6. Changes of PDCD4 mRNA in CCD-841-CoN and Caco-2 cells under long-term folic acid deficiency. GAPDH was used as an internal reference.

levels of miR-190 were decreased at 7 days and 21 days compared to the control. See **Figure 5**. In CCD-841-CoN cells, the mRNA level of miR-190 was decreased at 7 days ($P < 0.01$), but up-regulated at 21 days when comparing with the control (**Figure 5**). The results suggest that under folic acid deficiency, miR-200a plays different regulatory roles on miR-190 mRNA in normal colon epithelial cells and colorectal adenocarcinoma cells. The down-regulation of miR-200a and miR-190 mRNA in normal colonic epithelial cells at 7 days of folic acid deficiency may be the result of environment adaptation, which changes their metabolic state to better adapt to the living environment.

In order to further study the biological significance of miR-200a on miR-190, PDCD4 and PCNA were selected as parameters of cell apoptosis and cell proliferation, respectively. At 7 days, folic acid deficiency led to down-regulation of PCNA mRNA and PDCD4 mRNA in normal colonic epithelial cells. However, both PCNA and PDCD4 mRNA were up-regulated at 21 days. In contrast, PCNA mRNA was only down-regulated at 7 days in colorectal adenocarcinoma cells and up-regulated at 21 days, while PDCD4 mRNA remained down-regulated. See **Figures 6** and **7**. It is indicated that in a short-term folic acid deficiency state, miR-190 is reduced, so that mismatch repair mechanism cannot be started normally, and the body can reduce cell apoptosis by inhibiting proliferation of normal colon epithelial cells. The damage of cells can initiate apoptosis mechanism to further maintain intracellular homeostasis,

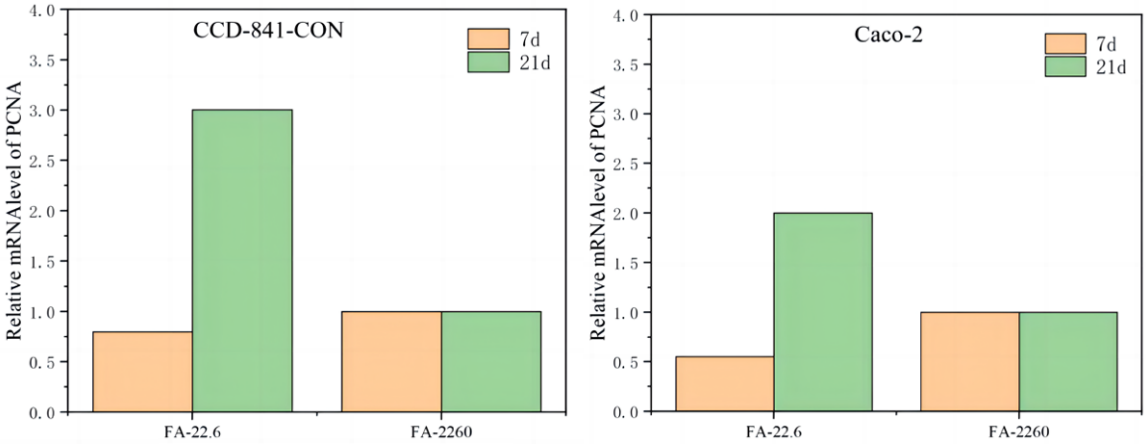


Figure 7. Changes of PCNA mRNA in CCD841-CoN and Caco-2 cells under long-term folic acid deficiency. GAPDH was used as an internal reference.

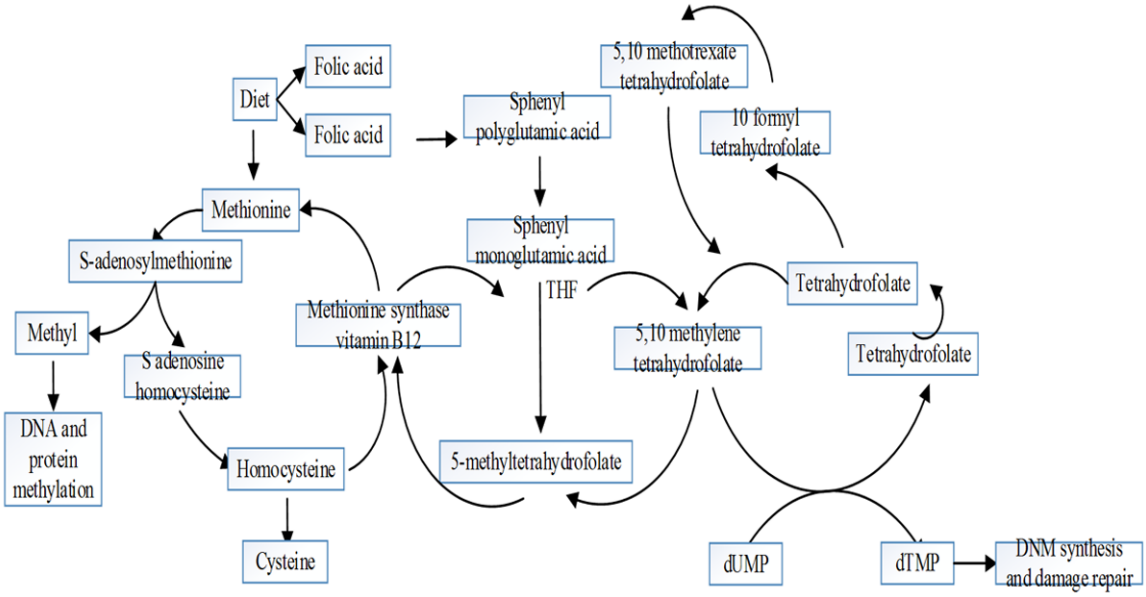


Figure 8. Metabolic process of folic acid in the body.

while the opposite phenomenon is shown in cancer cells, which may be one of reasons for tumorigenesis of normal cells.

Discussion

Dietary folic acid can be gradually converted into 5-methyltetrahydrofolate in the body. This 5-methyltetrahydrofolate is stored in liver through enterohepatic circulation and secreted into blood. The 5-methyltetrahydrofolate that enters body fluid circulation passes through various pathways such as folate receptors on the surface of tissue cell membranes and reduces folate carriers. Once inside the cells, 5-methyltetrahydrofolate undergoes two main metabolic pathways [12]. See **Figure 8**.

miRNA mainly regulates genes in following three ways: (1) complete complementarity with target gene mRNA transcript, which leads to the degradation of target gene mRNA; (2) incomplete complementarity with target gene, which inhibits protein translation; (3) by affecting the splicing pattern of the target gene mRNA [13]. See **Figure 9**.

The results of this study showed that folic acid deficiency led to genetic damage. Previous studies have shown that MTHFR is a key

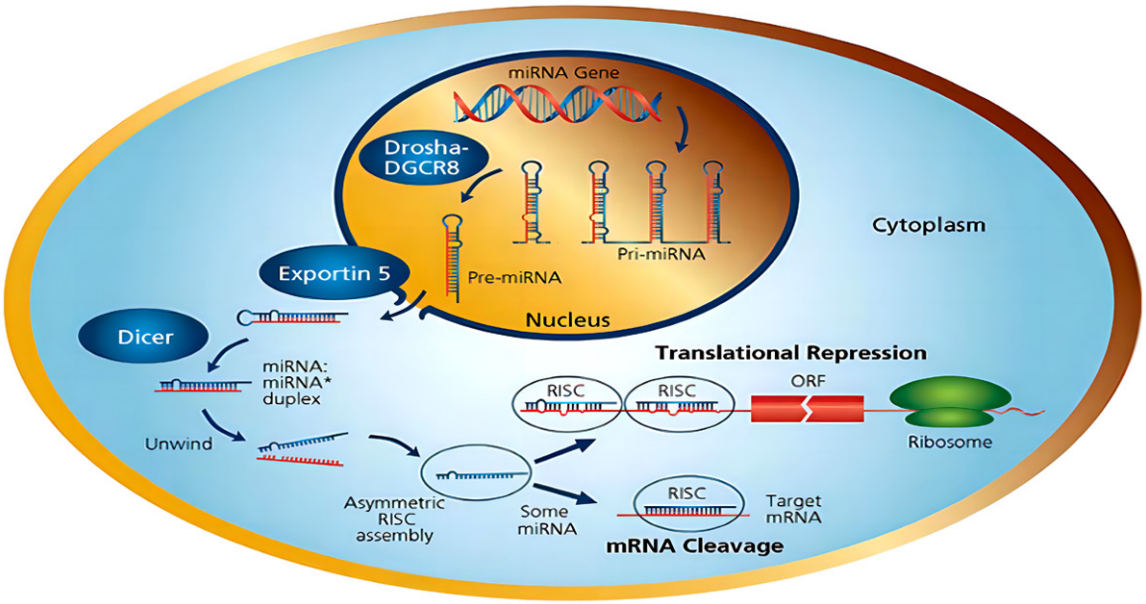


Figure 9. The process of miRNA maturation.

enzyme in folic acid metabolism, mainly catalyzing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is very important for the balance of DNA synthesis and DNA methylation enzymes. MTHFR enzyme activity affects the concentration of plasma 5,10-methylenetetrahydrofolate, and the concentration of 5,10-methylenetetrahydrofolate plays a key role in the conversion of dUMP to dTMP, affecting the synthesis and repair of DNA. The high concentration of 5,10-methylenetetrahydrofolate in cells is beneficial to the synthesis of dTMP and thus enhances the stability of DNA [14-16]. There is some evidence to suggest that a C→T substitution at the MTHFR gene 677 site may be associated with the risk of certain cancers. Specifically, individuals with the 677TT genotype may have a reduced risk of colon cancer, but an increased risk of gastric cancer and cervical cancer [17]. Studies have shown that individuals homozygous for the 677TT mutation have significantly higher plasma homocysteine levels compared to 677CC wild-type homozygous individuals, and significantly lower red blood cell folic acid concentrations than 677CC wild-type individuals [10]. Related reports also show that 677TT individuals may have a higher risk of breast cancer (or other related diseases) than 677CT and 677CC individuals [18]. Insufficient folic acid intake may also increase the likelihood of dUMP mispairing in DNA.

This study found that, at 7 days, human normal colonic epithelial cells CCD-841-CoN had no genome damage compared with normal controls, but at 21 days, folic acid deficiency increased the gene damage. In human colorectal adenocarcinoma cells Caco-2, the genome damage was gradually aggravated with the long-term deficiency of folic acid. Further analysis found that there was no difference in the MN between CCD-841-CoN and normal controls at 7 days, but at 21 days, the effect of folate deficiency on MNs was increased, and this phenomenon was also observed in Caco-2 cells at 21 days. More obviously, with the long-term deficiency of folic acid, MN continued to increase at 7 days and 21 days ($P < 0.01$). This phenomenon indicates that prolonged folic acid deficiency can lead to increased gene instability, such as DNA strand breaks and chromosome breaks, in both normal and cancer cells. Therefore, we believed that in vitro, low folic acid concentrations could increase genetic damage in human cells, which is consistent with our previous research results [14]. Given that the normal physiological concentration of folic acid in human plasma is 20-40 nmol/L, which is lower than the optimum folic acid concentration for maintaining genetic stability obtained by us, additional folic acid supplementation may help reduce the risk of human genetic damage and related diseases. Both miR-200a and miR-190 can be detected

in body fluids such as blood, urine, and cerebrospinal fluid, with good stability, making them potential candidates as serum tumor markers. It has been reported that miR-190 can inhibit tumor angiogenesis by reducing the expression of vascular endothelial growth factor and tumor growth [19]. miR-190 can act as a tumor suppressor gene and negatively regulate the expression of DPC4 gene in colorectal cancer cells. miR-200a, as a member of the miR-200 family, plays a key role in the proliferation, invasion and metastasis of tumor cells by regulating the EMT process [20]. This study suggested that miR-200a and miR-190 may play a vital role in the occurrence and progression of colorectal cancer. Studies have found that miR-200a and miR-190 were closely related to the occurrence of colorectal cancer, showing a high reference value in the early diagnosis of colorectal cancer. The results of this study showed that the expression of miR-200a was upregulated in human normal colonic epithelial cells CCD-841-CoN and colon cancer cells Caco-2 when folic acid was deficient for a long time. In human colorectal adenocarcinoma Caco-2, miR-200a was upregulated at 7 days and 21 days compared with that in the controls ($P < 0.01$). In normal colonic epithelial cells, miR-200a was down-regulated at 7 days but up-regulated at 21 days. The upregulation of miR-200a often occurs in malignant tumors or precancerous lesions, so long-term folic acid deficiency in normal cells may lead to the development of tumors in these cells.

Disclosure of conflict of interest

None.

Address correspondence to: Zhen Wang, Department of Colorectal Surgery, The Second Affiliated Hospital of Harbin Medical University, Harbin 150086, Heilongjiang, China. Tel: +86-13946052015; E-mail: zhenwangtg@yeah.net

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