• *GASTRIC CANCER* •

# Effects of folic acid on epithelial apoptosis and expression of Bcl-2 and p53 in premalignant gastric lesions

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## Abstract

**AIM:** To evaluate the effects of folic acid on epithelial apoptosis and expression of Bcl-2 and p53 in the tissues of premalignant gastric lesions.

**METHODS:** Thirty-eight patients, with premalignant gastric lesions including 18 colonic-type intestinal metaplasia (IM) and 20 mild or moderate dysplasia, were randomly divided into a treatment group ($n = 19$) receiving folic acid 10 mg thrice daily and a control group ($n = 19$) receiving sucralfate 1 000 mg thrice daily for 3 mo. All patients underwent endoscopies and four biopsies were taken prior to treatment and repeated after concluding therapy. Folate concentrations in gastric mucosa were measured with chemiluminescent enzyme immunoassay. Epithelial apoptosis and the expression of Bcl-2 and p53 protein in gastric mucosa were detected with flow cytometric assay.

**RESULTS:** The mean of folate concentration in gastric mucosa was $9.03 \pm 3.37$ μg/g wet wt in the folic acid treatment group, which was significantly higher than $6.83 \pm 3.02$ μg/g wet wt in the control group. Both the epithelial apoptosis rate and the tumor suppressor p53 expression in gastric mucosa significantly increased after folic acid treatment. In contrast, the expression of Bcl-2 oncogene protein decreased after folic acid therapy.

**CONCLUSION:** These data indicate that folic acid may play an important role in the chemoprevention of gastric carcinogenesis by enhancing gastric epithelial apoptosis in the patients with premalignant lesions.

**Key words:** Folic acid; Bcl-2; p53; Premalignant gastric lesions

## INTRODUCTION

Gastric cancer is one of the most common malignant diseases and remains the leading cause of cancer-related deaths in China[1]. Epidemiological analysis has revealed that individuals with a family history of gastric cancer have a 3-fold increased risk of developing gastric cancer as compared to the unaffected population[2,3], suggesting the fact that there is familial aggregation may reflect not only environmental but also genetic traits. Furthermore, nutritional studies have indicated that low consumption of fruits and vegetables is consistently related to an increased incidence of cancer[4]. Many components of fruits and vegetables such as folate and vitamin C may be responsible for the reduced risk of cancer[5-7]. It has been reported that diminished folate status is associated with cancer of the cervix, colorectum, lung, esophagus, brain, pancreas, and breast[8]. In a rat model of colonic carcinogenesis, folate deficiency enhances the development of colonic dysplasia and cancer, providing convincing evidence for the cause and effective relationship between diminished folate status and colon cancer[9]. Xiao *et al*[10] recently demonstrated that high-dose folic acid supplementation protected beagles against the development of gastric carcinogenesis induced by a chemical carcinogen N-ethyl-N-nitrosoguanidine.

Although the pathogenesis of gastric cancer still remains unclear, it has been proposed that the alteration of the balance between apoptosis and proliferation of gastric epithelial cells contributes to carcinogenesis[11]. The early indicator for the subject predisposed to gastric cancer is abnormal hyperproliferation of gastric epithelial cells, such as chronic atrophic gastritis, intestinal metaplasia (IM) and dysplasia, which have been considered as premalignant gastric lesions[12,13]. Generally, accumulation of genetic alterations including activation of oncogenes and inactivation of tumor suppressor genes plays an important role in the process from premalignant lesions to malignant transformation. However, little is known about the effects of folic acid on the expression of *bcl-2* oncogene and tumor suppressor *p53* in the premalignant gastric lesions. Therefore, the purpose of this study was to examine whether folic acid affects the apoptosis of epithelial cells and the expression

of Bcl-2 and p53 protein in the patients with IM and dysplasia.

## MATERIALS AND METHODS

### Patients

Patients with an endoscopic screening and histologically confirmed colonic type (grade III) IM and mild or moderate dysphasia by two or more expert pathologists on biopsy specimens were studied for this prospective, randomized, investigator-blind trial. Exclusion criteria were: (1) previous history of gastric surgery; (2) alcoholics; (3) current metabolic or life threatening disease; (4) recent use of vitamin supplements, methotrexate, triamterene, phenobarbital, pyrimethamine, trimethoprim, cholestyramine, sulfasalazine, or non-steroidal anti-inflammatory drugs; (5) pregnancy; and (6) anemia or vitamin $B_{12}$ deficiency. A total of 38 patients (18 IM and 20, dysplasia) who were recruited in the digestive unit at Affiliated Zhongda Hospital of Southeast University fulfilled the criteria for admission to the study. All patients gave their fully informed written consent before entering the study. The study also received the approval of the Medical Ethics Committee of Southeast University and Nanjing Medical University.

### Groups

The patients were randomly divided into a treatment group ($n = 19$) receiving folic acid tablet (Changzhou Pharmaceutical Factory, Changzhou, China) 10 mg thrice daily for 3 mo, and a control group ($n = 19$) receiving a gastric mucosal protective reagent, sucralfate tablet 1 000 mg thrice daily for 3 mo. Each group involves 9 IM and 10, dysplasia. Treatment group consisted of 13 men and 6 women with an average age of 50±7 years. Control group was made up of 14 men and 5 women with an average age of 49±9 years. No significant differences of sex ratio and average age were found between the two groups.

### Endoscopies and biopsy specimens

All endoscopic examinations were performed under local anesthesia with lidocaine. Four biopsy specimens (each 2 from the lesser and greater curvature of the antrum about 2 cm away from the pylorus respectively) were taken prior to folic acid treatment and repeated after concluding therapy.

### Measurement of folate concentrations in the gastric mucosa

Biopsy specimens were weighed and then homogenized in 0.2 mol/L acetic acid in a volume of 1 μL/μg. The homogenate was centrifuged at 1 500 $g$ for 15 min and the supernatants stored at -20 ℃ for the folate assay. The folate concentrations in the gastric mucosa were measured with chemiluminescent enzyme immunoassay kit according to the procedure indicated by the manufacturer (the IMMULITE 2000 system, DPC, Los Angeles, CA, USA). In brief, an aliquot of pretreated samples, ligand-labeled folate and folate binding protein (FBP) were added to the reaction tube containing a bead coated with a monoclonal antibody against FBP. Folate from the sample competed with the ligand-labeled folate for the FBP, and ligand-labeled folate-FPB complex bound to the anti-FBP antibody on

the bead. Alkaline phosphatase-labeled anti-ligand was added and bound to the ligand-labeled folate in the complex immobilized on the bead. Substrate addition initiated the chemiluminescent reaction, yielding a result that relates inversely to the folate concentrations in the samples. The concentrations of folate in gastric mucosa were expressed as micrograms of folate per gram tissue wet weight (μg/g wet wt).

### Assessment of cell cycle and apoptosis by flow cytometry analysis

For the preparation of gastric epithelial cell suspensions, biopsy specimens were treated for 30 min at 37 ℃ water bath with digesting solution containing 1% porcine pepsin A (by weight of substrate, and adjusted to pH 1.5 with 1 mol/L HCl). The reactions were terminated by adding 10 mL physiological saline. The cell solution was centrifuged at 750 r/min for 10 min and then pressed through a nylon cell strainer (Spectrum Laboratories Inc.) to isolate single-cell suspensions. Flow cytometry assay followed routine procedures by using $1 \times 10^6$ cells per sample.

The cell pellets were fixed with 200 μL of 70% ethanol at -20 ℃ for at least 4 h. After the ethanol was removed, the cells were washed once in PBS and then resuspended in 1 mL of propidium iodide/Triton X-100 staining solution [PBS containing 0.1% Triton X-100 (Sigma), 0.2 mg/mL RNAse A (Sigma), and 50 μg/mL propidium iodide (PI, Sigma)] and incubated for 30 min in the dark at room temperature. The DNA contents of the cells were analyzed by using a FACScan flow cytometer in combination with Cell Quest and ModFit LF software (Becton Dickinson, CA, USA). Apoptosis rates were based on the proportion of the peak area of the sub-G1 phase.

To confirm the type of cell death induced by folic acid, aliquots of $1 \times 10^6$ cells were washed in ice-cold PBS and suspended in binding buffer (Annexin V FITC kit, Immunotech, France). Annexin V fluorescein isothiocyanate (FITC) and PI were added to the cell suspension, and the mixture was incubated for 10 min on ice in the dark. The stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson). A minimum of 10 000 cells was counted in each sample; apoptosis rate represents the percentage ratio of Annexin V+/PI- cells to the total cell population.

### Flow cytometry analysis of Bcl-2 and p53 protein expression

For Bcl-2 and p53 expression, cells were stained with FITC-labeled monoclonal anti-Bcl-2 and anti-p53 antibodies (PharMingen, San Diego, CA, USA), washed, and stained with secondary antibodies. The samples were then run on a FACScan flow cytometer (Becton Dickinson), equipped with an argon laser emitting at 488 nm. A minimum of 10 000 cells were acquired in a list mode file format and analyses were performed with CELLQuest software (Becton Dickinson). The results were obtained as mean fluorescence index (MFI), calculated as the ratio of sample mean channel: control mean channel.

### Statistical analysis

Data were shown as mean±SD. Statistical analyses of matched data were performed using the Student's $t$-test. A

$P \leqslant 0.05$ was considered statistically significant.

## RESULTS

### Folate concentrations in the gastric mucosa

Three months after folic acid treatment, the mean of folate concentrations in gastric mucosa was ($9.03 \pm 3.37$) μg/g wet wt in the treatment group, which were significantly higher than ($6.83 \pm 3.02$) μg/g wet wt in the control group ($P<0.01$ Table 1).

**Table 1** Changes in the gastric mucosal folate concentrations after folate treatment

| Group | $n$ | Pre-T | Post-T |
|---|---|---|---|
| Treatment | 19 | 7.16±1.88 | 9.03±3.37[b] |
| Control | 19 | 6.90±2.72 | 6.83±3.02 |

$n$: number; Treatment: treatment group; Control: control group; Pre-T: before treatment; Post-T: after treatment; Data are shown as (mean±SD) μg/g wet wt. [b]$P<0.01$ *vs* control group and before treatment in the same group.

### Cell cycle analysis and apoptosis

Figure 1 shows the distributions of the DNA contents in gastric epithelial cells after folic acid treatment. A high peak in the sub-G1 phase as an induction of apoptosis was detected after folic acid therapy. However, the effect was not observed in the control group treated with sucralfate (data not shown).
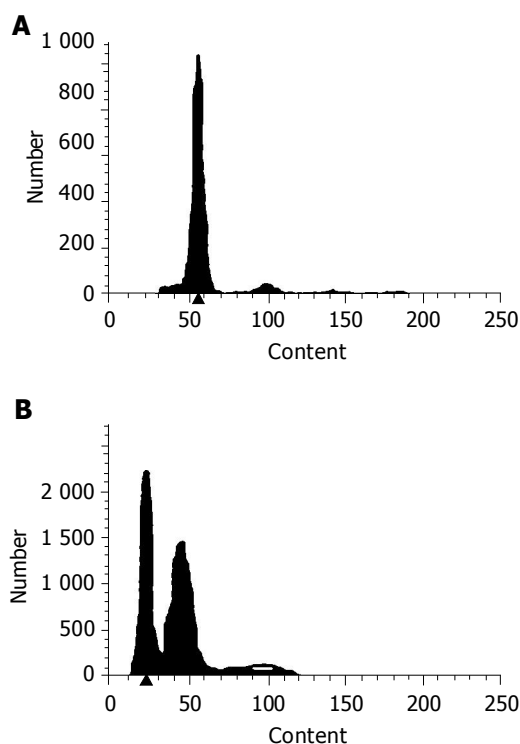
To confirm the type of cell death induced by folic acid, gastric epithelial cells were stained twice with PI and FITC-labeled Annexin V, and then analyzed by flow cytometry (Table 2). As Annexin V detects phosphatidylserine exposure in the plasma membrane of apoptotic cells, this technique can differentiate among normal (double negative), early apoptotic (Annexin V-FITC single positive), and necrotic (Annexin V-FITC and PI double positive) cells. In Figure 2, the lower left population of cells that have low level of FITC and PI signals in each plot indicates normal cells. Those in the upper left, which have low FITC and high PI signals, indicate necrotic cells. The lower right and upper right populations correspond to apoptotic and secondary necrotic (late apoptotic), which have high FITC and low PI, and high FITC and high PI signals respectively. More than 80% of

**Table 2** Effects of folic acid on the apoptosis rate in gastric epithelial cells

| Group | $n$ | Staining with PI (%) | | Staining with annexin V FITC/PI (%) | |
|---|---|---|---|---|---|
| | | Pre-T | Post-T | Pre-T | Post-T |
| Treatment | 19 | 6.19±2.82 | 6.78±2.15[b] | 16.22±3.80 | 21.00±4.61[a] |
| Control | 19 | 6.27±3.24 | 6.23±2.70 | 15.10±2.52 | 15.70±2.56 |

Apoptosis rate represents the ratio of Annexin V+/PI- cells to the total cell population. $n$: number; Treatment: treatment group; Control: control group; Pre-T: before treatment; Post-T: after treatment; Data are shown as (mean±SD)%. [a]$P<0.05$ and [b]$P<0.01$ *vs* control group and before treatment in the same group.



**Figure 1** Effect of folic acid on the cell cycle and the induction of apoptosis DNA content was determined by flow cytometry after ethanol fixation, permeabilization and PI staining. A: before; B: after folic acid treatment. Representative results of one of the subjects are shown.
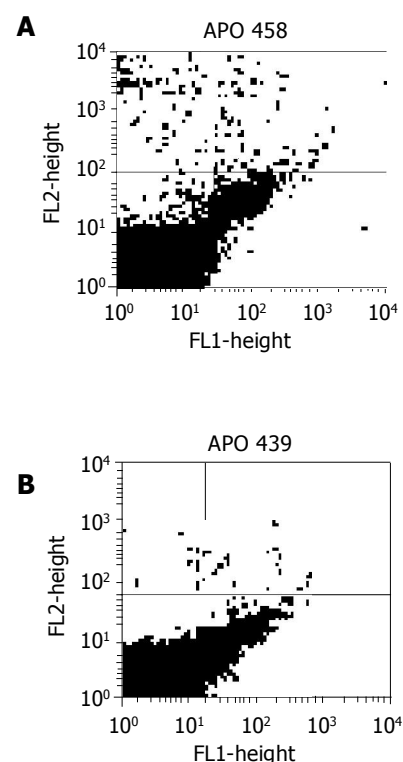


**Figure 2** Annexin V-FITC/propidium iodide double staining of gastric epithelium before and after treatment with folic acid The proportion of apoptosis was measured by flow cytometry using Annexin V-FITC and PI. In the figures, X-axis (FL1-H) indicates Annexin V, Y-axis (FL2-H) indicates PI. A: before; B: after folic acid treatment. Representative results of one of the subjects are shown.

the untreated cells were Annexin and PI double negative (Figure 2A), whereas an increase in the lower right population was observed (Figure 2B) after folic acid treatment.

### Expression of Bcl-2 and p53 protein

We also investigated intracellular expression of molecules known to be involved in apoptosis such as Bcl-2 and p53. Representative distributions of Bcl-2 and P53 in the gastric epithelial cells were shown in Figure 3 (A-D). Bcl-2 MFI was weaker whereas P53 MFI was higher after folic acid treatment (B and D) when compared with treatment before (A and C). Therefore, after treatment with folic acid for 3 mo, expression of Bcl-2 decreased and that of p53 increased significantly (Table 3).

**Table 3** Effects of folic acid on the expression of Bcl-2 and p53 in gastric epithelial cells

| Group | $n$ | Bcl-2 expression | | p53 expression | |
|---|---|---|---|---|---|
| | | Pre-T | Post-T | Pre-T | Post-T |
| Treatment | 19 | 8.17±2.06 | 6.22±1.26[a] | 2.03±0.35 | 2.56±0.47[b] |
| Control | 19 | 6.78±2.57 | 6.98±2.55 | 1.75±0.65 | 1.70±0.39 |

$n$: number; Treatment: treatment group; Control: control group; Pre-T: before treatment; Post-T: after treatment; Data are shown as (mean±SD)%. [a]$P<0.05$ and [b]$P<0.01$ $vs$ control group and before treatment in the same group.

## DISCUSSION

Folate is a member of water-soluble B vitamin family; its principal biochemical function is the mediation of one-carbon transfer or methylation reactions. These reactions include (1) purine and pyrimidine nucleotide biosynthesis; (2) amino acid conversions-the interconversion of serine and glycine, catabolism of histidine to glutamic acid and conversion of homocysteine to methionine; (3) generation and utilization of formate and (4) methylation of small amounts of transfer RNA. Folate deficiency has been shown to be common in various regions of China including Beijing[14,15], and a number of epidemiological studies conducted in the Chinese population have consistently indicated an inverse association between consumption of vegetables and fruits, a major source of folate, and the risk of gastroesophageal cancer[16-18]. Folic acid is the synthetic form of folate, which is used for nutritional supplements and food fortification. The present study demonstrated that folic acid, which was administered to the patients for 3 mo, markedly increased the concentrations of folate in the gastric mucosa. A higher intake of the micronutrient folate was first proposed by Freudenheim[19] to reduce the risk of colorectal cancer. Thereafter, the evidence from epidemiological, animal and human studies strongly suggests that folate status modulates the risk of several malignancies, the most notable of which is the colorectum[8,9,20-22]. Recent animal study indicated that high dose of folic acid also played a prevention role in gastric carcinogenesis in beagles[10].

Although the incidence rates for gastric cancer have been declining in many countries[23], it remains the leading cause of cancer-related deaths in China[1,24]. The incidence and mortality of gastric cancer varies from province to province, generally very high in the north but relatively low in the south of China[25], suggesting that environmental factors, particularly those associated with the diet, may play an important role in gastric carcinogenesis. More than 90% of gastric cancers are adenocarcinomas, which are divided into intestinal and diffuse histological types. In the view of
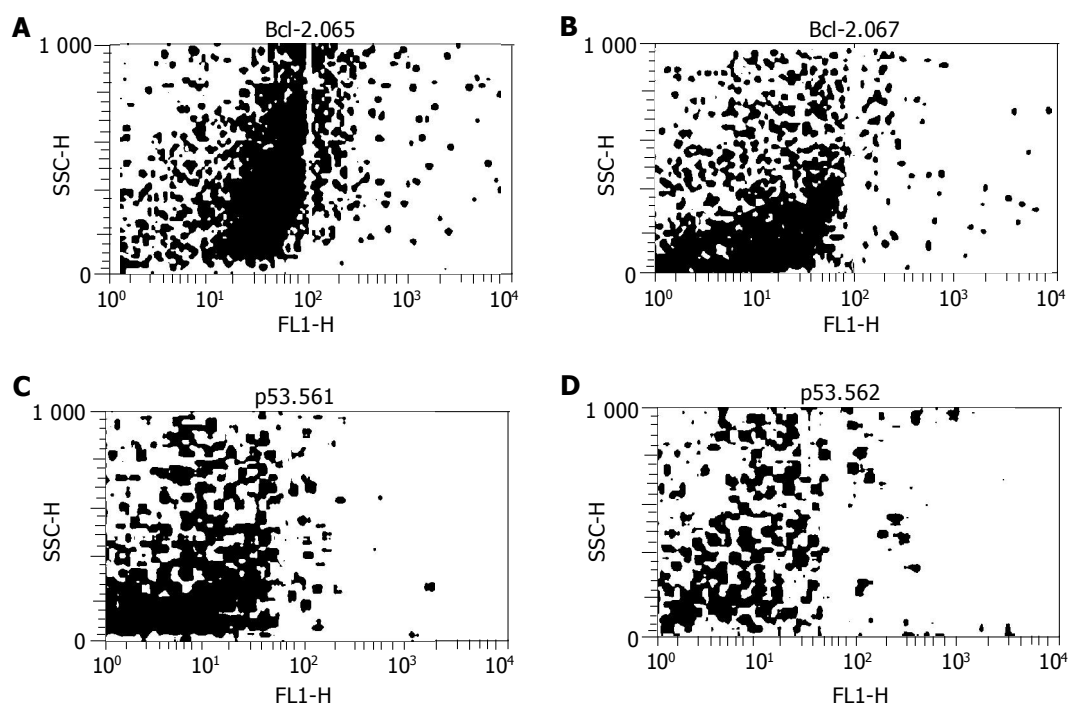


**Figure 3** Flow cytometry analysis of Bcl-2 and p53 expression in a patient before and after folic acid treatment For Bcl-2 and p53 expression, cells were stained with FITC-labeled monoclonal anti-Bcl-2 (A and B) or anti-p53 antibodies (C and D); and then measured by flow cytometry. A and C: before; B and D: after folic acid treatment. Representative results of one of the subjects are shown.

Correa's multistep model of gastric carcinogenesis[13,26], intestinal-type gastric cancer develops from chronic active gastritis, premalignant lesions (chronic atrophic gastritis, IM and dysplasia) and finally to gastric cancer. Therefore, studying the effects of interventions on the progression of these premalignant lesions is the most viable option for decreasing the risk of gastric cancer. It has been demonstrated that folic acid supplementation reverses IM and dysplasia and thus delays the progression of gastric carcinogenesis[27]. Mark and colleagues[28] have also revealed the reversion effect of vitamin and mineral supplement including folate on esophageal dysplasia. However, the mechanisms for these phenomena have not yet been clarified.

Folate is an important factor in DNA synthesis, stability and integrity, repair and methylation, aberrations all of which are implicated in carcinogenesis[8,29,30]. A growing body of *in vivo* and *in vitro* evidence suggests that folate deficiency is associated with DNA damage, impaired DNA repair, abnormal DNA methylation, and increased susceptibility to mutagenesis, which can be overcome by folate supplementation[8,30]. An alteration in DNA methylation has been suggested to be an important factor in causing genetic instability[31,32] and is thought to contribute to carcinogenesis by affecting the expression of proto-oncogenes and/or tumor suppressor genes[33]. The present study clearly shows that folic acid significantly increases the epithelial apoptosis and p53 expression in the gastric mucosa. The tumor suppressor gene *p53* may directly induce apoptosis through several pathways and plays a major role in the protection of cells from DNA damage[34,35]. The balance between cell proliferation and apoptosis results in a disturbance of tissue homeostasis and this may promote the development of cancer. Recent insight into the *p53*-mediated biochemical pathways of cell-cycle arrest and apoptosis has provided further understanding of the mechanisms related to *p53*-mediated tumor suppression[36]. The transformation of gastrointestinal epithelial tissue to carcinomas has been shown to be associated with the progressive inhibition of apoptosis[37,38]. In the present study, high levels of p53 protein from intestinal metaplastic and dysplastic epithelium were induced by folic acid, and overexpression of p53 could arrest the cell cycle and induce apoptosis in high-risk patients for gastric cancer.

Apoptosis is an essential and highly conserved mode of cell death that is important for normal development, host defense and suppression of oncogenesis. Among the numerous proteins and genes involved, members of the Bcl-2 family play a central role to inhibit or promote apoptosis[39]. Levels of Bcl-2 within cells are critical to antiapoptotic activity, decreasing Bcl-2 could be a mechanism to sensitize cells to apoptosis[39,40]. The present study has demonstrated that folic acid was able to induce the apoptosis in premalignant gastric lesions; this apoptosis may be mediated by down-regulating the expression of apoptosis-associated gene *bcl-2* and up-regulating the expression of tumor suppressor gene *p53*. Apoptosis is distinguished from necrosis because it is highly regulated, requires new gene expression, and leads to changes in nuclear morphology, DNA laddering and membrane blabbing[41]. The changes in membrane composition lead to extracellular exposure of phosphatidylserine (PS) residues and occur early in the apoptotic cycle, regardless of the initiating signal[42]. Exposed PS residues avidly bind Annexin V, a natural ligand, in a calcium-dependent manner[43]. Membrane changes leading to PS exposure occur rapidly in apoptotic cells, while the cell loses membrane integrity later in the apoptotic process. Necrotic cells expose PS and lose membrane function simultaneously soon after cell injury[43]. Using a DNA binding dye such as PI in tandem with fluorochrome-conjugated Annexin V, apoptotic cells are identified and discriminated from necrotic cells[42]. Because the extracellular exposure of PS occurs earlier than DNA fragmentation in the apoptotic cycle, Annexin V-FITC/PI double staining is superior to other apoptosis detection methods, such as PI staining and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling) assay.

Furthermore, folic acid is important for the production of S-adenosylmethionine, the primary methyl donor for DNA methylation[29,44]. Decreased DNA methylation is associated with an increased risk of human gastric cancer[45]. Folate deficiency may deplete cellular S-adenosylmethionine levels causing DNA hypomethylation and inappropriate activation of proto-oncogenes[46].

In conclusion, this study demonstrates that both the epithelial apoptosis rate and the tumor suppressor p53 expression in gastric mucosa were significantly increased, while the expression of *Bcl-2* oncogene protein decreased after folic acid treatment. These findings indicate that folic acid may play an important role in the chemoprevention of gastric carcinogenesis by enhancing gastric epithelial apoptosis in patients with premalignant lesions.

## ACKNOWLEDGEMENTS

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