

Folic acid mediated attenuation of loss of heterozygosity of *DCC* tumor suppressor gene in the colonic mucosa of patients with colorectal adenomas

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Abstract

Loss of heterozygosity (LOH) and/or inactivation of tumor suppressor genes are implicated in the initiation and progression of many malignancies, including colorectal cancer. Although accumulating evidence suggests a chemopreventive role for folate in colorectal cancer, regulatory mechanisms are poorly understood. The primary objective of the current investigation was to determine whether folic acid would prevent LOH of the three tumor suppressor genes, deleted in colorectal cancer (*DCC*), adenomatous polyposis coli (*APC*) and *p53* in macroscopically normal appearing rectal mucosa of patients with adenomatous polyps. In addition, the effect of folic acid on rectal mucosal proliferation was determined. Twenty patients were randomized in a double-blind study to receive either folic acid 5 mg once daily or identical placebo tablets for 1 year. Genomic DNA and total protein were extracted from the rectal mucosa at baseline and after 1 year of treatment and analyzed for LOH and protein levels of *APC*, *DCC* and *p53* genes. In addition, paraffin-embedded mucosal specimens were analyzed for proliferating cell nuclear antigen (PCNA) immunoreactivity, as a measure of cellular proliferative activity. Folate supplementation prevented LOH of *DCC* gene in five out of five (100%) patients who demonstrated baseline heterozygosity, whereas two out of four (50%) placebo-treated patients with baseline heterozygosity demonstrated allelic loss. Mucosal protein levels of *DCC* were also reduced in 7 of 10 (70%) placebo-treated patients compared to only 2 of 10 (20%) of patients treated with folate. Levels increased, however, in eight and three patients in the folic acid and placebo groups, respectively ($P < 0.02$). Folic acid caused no change in allelic status of either *APC* or *p53* gene. Folate supplementation caused a small, but not statistically significant, 16% reduction in mucosal proliferation, whereas placebo treatment resulted in a 88% ($P < 0.05$) increase in this parameter, when compared with the corresponding baseline values. Our results indicate that folic acid prevents an increase in proliferation and arrests LOH of *DCC* gene and also stabilizes its protein in normal appearing rectal mucosa of patients with colorectal adenomas. Taken together, our data suggest that one of the ways folate may exert its chemopreventive effect is by stabilizing certain tumor suppressor gene(s) and preventing further increases in proliferation.

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1. Introduction

Although the epidemiology of colorectal cancer is clearly related to genetic susceptibility, dietary factors such as

vitamins and micronutrients are thought to influence tumorigenic processes [1,2]. Over the past several years, considerable evidence has appeared that suggests a role for the water-soluble Vitamin folic acid in reducing colorectal carcinogenesis [3]. Several epidemiological studies have reported an inverse relationship between dietary intake or blood folate levels and the incidence of colorectal cancer [4–7]. Additionally, it has been reported that folate levels are lower in adenoma and carcinoma of the colon than in normal

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appearing mucosa [8], suggesting a role for mucosal folate in the development and progression of colorectal cancer. In experimental models of colonic carcinogenesis, folate deficiency was found to augment the dimethylhydrazine-induced colorectal tumors in rats [3], whereas folate supplementation protects from the development of colonic neoplasms in a dose-dependent manner [9]. In the azoxymethane-induced colon cancer rat model [10], supplemental folic acid has also been shown to decrease the formation of aberrant crypt foci, which are considered to be precursor of colorectal adenomas and carcinoma [11,12]. Recent *in vitro* studies from this and other laboratories have further demonstrated that supplemental folic acid greatly inhibits proliferation of colon cancer cell lines [13,14]. However, the mechanisms by which folic acid exerts its chemopreventive role in colorectal carcinogenesis are poorly understood. Carcinogenesis, which is a multi-step process, results from the accumulation of mutations during progression from normal epithelium to carcinoma [15]. The mechanisms of gene inactivation include allelic deletion (loss of heterozygosity or LOH), chromosomal rearrangement, point mutation and inactivation of suppressor gene products by viral or cellular inactivation agents. Genetic changes that occur at different stages of epithelial cell carcinoma have been extensively studied by Vogelstein and his colleagues in human colon cancer [16]. Inactivation of a number of tumor suppressor genes, including adenomatous polyposis coli (*APC*), deleted in colorectal cancer (*DCC*) and *p53* has been detected in the development and progression of colorectal cancer [16–19]. However, it remains to be determined whether mutational status of these and other tumor suppressor genes in the colorectal mucosa might be affected by supplemental folic acid. The present pilot study was, therefore, undertaken to determine the changes in mutational status of *APC*, *DCC* and *p53* genes in macroscopically normal appearing rectal mucosa at the beginning (baseline, i.e. before treatment) and 1 year after treatment with either supplemental folic acid or placebo tablets. Furthermore, since hyperproliferation is considered central to the development and progression of gastrointestinal carcinogenesis [20], changes in mucosal proliferative activity were also determined in these subjects before and after the treatment with folic acid or placebo.

2. Materials and methods

2.1. Study design and subjects

Male or female subjects aged >18 years undergoing colonoscopy who were found to have at least one adenoma >0.5 cm in diameter were eligible for study entry. All subjects were required to have adequate nutritional status as well as normal renal and liver function tests. We excluded subjects with a family history of colorectal cancer, severe co-morbid conditions involving the heart or lungs, inflammatory bowel disease, a history of gastric or intesti-

nal surgery, pernicious anemia, low serum Vitamin B12, abnormal serum or RBC folate, or a previous history of colorectal cancer. Patients regularly ingesting or requiring steroids, non-steroidal anti-inflammatory drugs (NSAIDs), antineoplastic drugs, folate antagonists, drugs known to antagonize folate, or multivitamins were also excluded. We further excluded any patients who had regularly consumed multivitamins, folic acid, aspirin or NSAIDs for a cumulative period of ≥ 3 months throughout the year prior to study entry. Patients were encouraged to take acetaminophen as needed for pain and to refrain from NSAID/aspirin use throughout the study period. Patients consuming more than two alcoholic drinks daily or with a history of active or previous illicit drug abuse were also excluded.

Subjects who successfully completed a 4-week placebo run-in period by consuming at least 75% of their prescribed medication were randomly assigned to receive either one 5 mg folic acid tablet (Stanley Pharmaceutical, Toronto, Canada) or one identical placebo tablet once daily in a double-blind fashion. All patients had a complete history and physical examination performed by a gastroenterologist and a detailed nutritional assessment prior to study entry. Patients consuming <75% of their prescribed medication were excluded from final analysis. All subjects had all baseline colorectal polyps removed and categorized according to size, location and histology. Additionally 10 forceps mucosal biopsies were obtained from macroscopically normal appearing tissue in the rectum (<10 from anal verge), two of which were immediately fixed in 10% buffered formalin for histological and immunohistochemical studies and the remainder were snap frozen and stored at -90°C .

Subjects were evaluated at 3, 6 and 12 months with pill counts to assess compliance and for assessment of any adversities to treatment. All patients had a serum folate and RBC folate repeated after 3 months of study entry. All patients had sigmoidoscopy performed at 1 year specifically to examine the rectum and to obtain 10 forceps mucosal biopsies from macroscopically normal appearing mucosa (<10 cm from the anal verge) for comparative analyses.

2.2. Determination of proliferating cell nuclear antigen immunoreactivity (PCNA)

This was performed as described previously [21]. Briefly, serial sections (4 μm thick) were deparaffinized and incubated in ambient temperature with anti-PCNA (monoclonal antibody) for 30 min at 1:50 dilution. The avidin–biotin technique was then performed with matched components (secondary biotinylated antibody and avidin–peroxidase complex) from the DAKO labeled streptavidin–biotin system (Carpinteria, CA) according to the manufacturer's suggested protocol. Amino ethyl carbazole was used as chromagen to localize PCNA-positive cells. All slides were slightly counterstained with Harris' hematoxylin and examined by a pathologist blinded to sample coding. At least 10 well-oriented crypts on each slide and five slides from each

sample were examined under high power. At least 750 cells per slide were counted using a 40× objective.

2.3. DNA extraction

Genomic DNA was extracted from each biopsy specimen using DNA STAT-60 reagent (Tel-Test Inc., TX), according to manufacturers instruction. Biopsy samples were homogenized in DNA STAT-60 reagent and DNA was precipitated from the aqueous phase using isopropanol.

2.4. Detection of LOH of APC

LOH of APC was studied according to the procedure described by Tandle et al. [22]. This method involves studying restriction fragment length polymorphism (RFLP) of APC in exon 11 by digesting with RsaI following PCR amplification. Sample DNA template in the PCR reaction was denatured first at 94 °C for 1.5 min and the target gene amplified at 94 °C for 40 s, 59 °C for 40 s and 72 °C for 40 s for 35 cycles followed by 15 min of extension at 72 °C. The primers were used were sense 5'-GGACTACAGGCCATTGCAGAA-3' and antisense 5'-GGCATCATCTCCAAAAGTCAA-3'. The amplified 133 bp fragment was then digested with RsaI (NEB) and products analyzed on 2.5% agarose gels.

2.5. Detection of LOH of p53

LOH in p53 was studied essentially according to the procedure described by Ara et al. [23]. This method utilizes restriction fragment length polymorphism exhibited by codon 72, which can be detected by restriction digestion with BstUI, following PCR amplification. Template DNA in the PCR reaction was denatured at 94 °C for 1.5 min and the target gene amplified at 94 °C for 40 s, 60 °C for 40 s and 72 °C for 45 s for 40 cycles followed by extension at 72 °C for 15 min. The primers used were sense 5'-TTGCCGTCCCAAGCAATGGATGA-3' and antisense 5'-TCTGGGAAGGGACAGAAGATGAC-3' to amplify a 200 bp fragment. PCR products were the digested with BstUI (New England Biolabs Inc., Beverly, MA) and separated on 2.5% agarose gels (FMC Bioproducts).

2.6. Detection of LOH of DCC

To study LOH of DCC, variable number of tandem repeats (VNTR) within the DCC gene [24,25] was amplified. PCR reactions containing sample DNA template were denatured at 94 °C for 1.5 min and the target gene amplified at 94 °C for 30 s, 58 °C for 40 s and 72 °C for 40 s for 40 cycles followed by 15 min of extension at 72 °C. The primers used were sense 5'-GATGACATTTCCCTCTAG-3' and antisense- 5'-GTGGTTATTGCC TTGAAAG-3' [21]. The PCR products were separated on 2.5% agarose gels (FMC).

2.7. Western blot

The relative concentration of DCC protein was analyzed by Western blot as described previously [26]. Briefly, each biopsy specimen was homogenized in 0.1 ml of lysis buffer (50 mM Tris pH 7.4, 100 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 0.5% NP-40, 2.5 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml aprotinin and leupeptin, 50 µg/ml Soybean trypsin inhibitor). The homogenate was rotated for 30 min at 4 °C and subsequently centrifuged at 11,000 × g for 15 min at 4 °C, and the supernatant used in all experiments. Protein concentration was measured using the protein assay kit from Bio-Rad laboratories.

The 11,000 × g supernatant containing 50 µg of protein was separated on a 7.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted to a nitrocellulose membrane. The membrane was blocked overnight with 5% non-fat dried milk in TBS-T buffer (20 mM Tris, pH 7.6, 100 mM NaCl, 0.1% Tween 20), followed by 3 h of incubation with the primary antibody (DCC monoclonal antibody; Oncogene research; 1:1000 dilution) in TBS-T buffer containing 5% non-fat dried milk at room temperature. After washing three times with TBS-T buffer, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG as a second antibody (1:5000 dilution) for 1 h at room temperature. Proteins were visualized using by enzyme-linked enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL). The membranes were stripped and then probed with β-actin antibodies (Boehringer-Mannheim) as an internal control. Signals on the blots were visualized by autoradiography and quantitated by densitometry using ImageQuant image analysis system (Storm Optical Scanner, Molecular Dynamics, Sunnyvale, CA).

2.8. Statistical analysis

Comparison of baseline patient characteristic were analyzed for intersubject variation using the unpaired Student's *t*-test. Data examining changes in mucosal proliferation from baseline were analyzed using a 2 × (2) [group × (time)] mixed design analysis of variance determining test and retest as within subjects dimensions and treatment groups as between subjects dimensions. Analysis of DCC levels were compared using a test of significance of difference between two proportions according to Bruning and Kintz. Unless otherwise stated data are expressed as the mean ± S.D. *P* value <0.05 was designated as the level of significance.

3. Results

3.1. Characteristics of the subjects

Twenty eligible subjects who successfully completed a 4-week placebo run-in period were randomized to receive

Table 1
Baseline characteristics of subjects

Characteristics	Treatment groups		P-value
	Folate (n = 10)	Placebo (n = 10)	
Age (years)	54.7 ± 7.08	61.8 ± 8.3	NS
Sex (male)	9/10	10/10	
Current smokers	6/10	7/10	NS
Dietary intake			
Total fat (g per day)	89.11	88.1	NS
Fiber (g per day)	6.2 ± 5.2	6.0 ± 3.5	NS
Folate (µg per day)	128.4 ± 86.4	165.2 ± 106	NS
Calcium (mg per day)	727.2 ± 379	665.3 ± 314	NS
Number of aspirin users (≤ 325 mg per day)	0/10	2/10	NS
History of adenoma	3/10	3/10	NS
Adenomas			
Size (mm)	10.7 ± 7.1	11.6 ± 7.5	NS
Number	1.6 ± 0.96	2.5 ± 1.64	NS
No. villous histological findings	1/10	1/10	NS

either folic acid ($n = 10$) or identical placebo ($n = 10$). Table 1 shows the baseline characteristics and nutritional assessment results of all randomized subjects at entry which were similar between the treatment groups. Serum and RBC folic acid levels obtained after 3 months of folate supplementation were significantly increased in the folic acid treatment arm compared to baseline and to 3-month values in placebo-treated patients; no significant difference was observed between the baseline and 3-month values in placebo-treated patients (Table 2). Baseline liver and renal functions were normal in all patients (data not shown). All 20 patients completed the 1-year trial and satisfied compliance requirements by consuming >75% of the prescribed medication. Treatment was well tolerated by all subjects and no patients reported any adverse effects from folate supplementation throughout the 1-year study period. Two patients in the placebo group admitted to taking daily aspirin 81 and 325 mg, respectively, during the study period. All the remaining patients denied ASA or NSAID use and

Table 2
Baseline and 3 months folate serologies

	Treatment groups		P-value
	Folic acid folate levels (ng/ml)	Placebo folate levels (ng/ml)	
Baseline			
Mean serum folate	8.89 ± 4.25	9.64 ± 3.71	NS
Mean RBC folate	390 ± 117	508 ± 126	NS
After 3 months of treatment			
Mean serum folate	133 ± 120*	14.4 ± 14**	<0.018
Mean RBC folate	1033 ± 211*	486 ± 159**	<0.001

* $P < 0.001$, compared to baseline.

** $P = NS$, compared to baseline.

admitted to using acetaminophen periodically throughout the study period.

3.2. Changes in LOH of tumor suppressor genes

Loss of heterozygosity and/or inactivation of tumor suppressor genes are implicated in the initiation and progression of colorectal cancer [16,27]. To determine whether folic acid might prevent LOH of tumor suppressor genes, mutational status of *APC*, *DCC* and *p53* genes were analyzed in macroscopically normal appearing rectal at the beginning (baseline, i.e. before treatment) and after 1 year of treatment with either supplemental folic acid or placebo tablets. Results are shown in Table 3 and Fig. 1. Of 10 polypectomized subjects, who consumed supplemental folic acid for 1 year, five were homozygous for *DCC* gene with 160 bp allele and five were heterozygous. Four of the heterozygotes showed 200 and 160 bp alleles, and one had alleles that were slightly higher than 200 bp and lower than 160 bp. Whether the slight differences in size of amplified DNA is due to insertions or deletions remains to be determined. We observed that folic acid supplementation prevented LOH of *DCC* gene in five out of five patients (100%) who demonstrated baseline heterozygosity (Table 3; subjects # 11–13, 18, and 19), whereas two out of four (50%) placebo-treated patients with baseline heterozygosity (Table 1; subjects # 2, and 3) demonstrated complete allelic loss. In contrast to what we have observed for *DCC*, supplemental folic acid caused no change in allelic status of either *APC* or *p53* genes in polypectomized patients (Table 3). No change in mutational status of either *APC* or *p53* gene was observed among the polypectomized subjects, who were given placebo tablets (Table 3).

In selected samples, including those with LOH in the *DCC* gene, expression of DCC protein was also examined. The levels of DCC protein in the rectal mucosa from placebo-treated subjects, two of whom demonstrated complete allelic loss (subjects # 2, and 3) and one (subject # 1) with no allelic loss, were found to be substantially lower when compared with the corresponding baseline values (Figs. 2 and 3). In addition, colonic mucosal DCC levels in four other subjects (subjects # 4, 5, 8, and 9) were decreased, compared to the corresponding baseline levels (Figs. 2 and 3). In contrast, in folic-acid treated subjects, rectal mucosal DCC protein levels were either found to be higher (20–70%) in five subjects (subjects # 14, 15, 17, 18, and 20) or remained essentially unchanged in three subjects (subjects # 11, 12, and 16) over the corresponding baseline values ($P < 0.02$) (Figs. 2 and 3).

3.3. Changes in mucosal proliferation

Hyperproliferation is considered to be central to the development of carcinogenesis in the gastrointestinal tract [20]. Since *DCC* gene has been implicated in the progression of colorectal cancer [28,29], we postulated that our

Table 3

Changes in LOH of *DCC* gene in macroscopically normal rectal mucosa at the beginning (baseline, i.e. at the time of polypectomy) and 1 year after treatment with supplemental folic acid or placebo tablets in polypectomized patients

Subjects	Baseline			After 1 year		
	DCC (VNTR)	APC	p53	DCC (VNTR)	APC	p53
				Placebo		
1	Het	Hom (n)	Hom (c)	Het	Hom (n)	Hom (c)
2	Het	Hom (n)	Het	LOH	Hom (n)	Het
3	Het	Hom (n)	Het	LOH	Hom (n)	Het
4	Hom	Het	Het	Hom	Het	Het
5	Hom	Het	Het	Hom	Het	Het
6	Hom	Het	Het	Hom	Het	Het
7	Het	Hom (n)	Het	Het	Hom (n)	Het
8	Hom	Het	Het	Hom	Het	Het
9	Hom	Het	Het	Hom	Het	Het
10	Hom	Het	Het	Hom	Het	Het
				Folic acid		
11	Het	Het	Hom (c)	Het	Het	Hom (c)
12	Het	Hom (n)	Hom (c)	Het	Hom (n)	Hom (c)
13	Het	Het	Hom (c)	Het	Het	Hom (c)
14	Hom	Het	Het	Hom	Het	Het
15	Hom	Hom (n)	Het	Hom	Hom (n)	Het
16	Hom	Hom (n)	Het	Hom	Hom (n)	Het
17	Hom	Hom (n)	Het	Hom	Hom (n)	Het
18	Het	Het	Het	Het	Het	Het
19	Het	Het	Hom (c)	Het	Het	Hom (c)
20	Hom	Het	Hom (c)	Hom	Het	Hom (c)

Het: heterozygous, Hom: homozygous, Hom (n): homozygous uncut allele, Hom (c): homozygous cut allele, LOH: loss of alleles. All the informative cases for DCC (VNTR) are indicated in bold.

observation of prevention of LOH of *DCC* gene by folic acid might lead to suppression of rectal mucosal proliferation. To test this hypothesis, we examined the changes in PCNA immunoreactivity in the rectal mucosa as a measure of proliferative activity. The 2 × (2) mixed ANOVA revealed

a significant group × (time) interaction [$F(1, 23) = 9.52$; $P < 0.005$]. Baseline values of mucosal proliferation did not significantly differ between the groups. In polypectomized patients, daily supplementation with 5 mg folic acid for 1 year resulted in a 16% reduction ($P = NS$) in the number

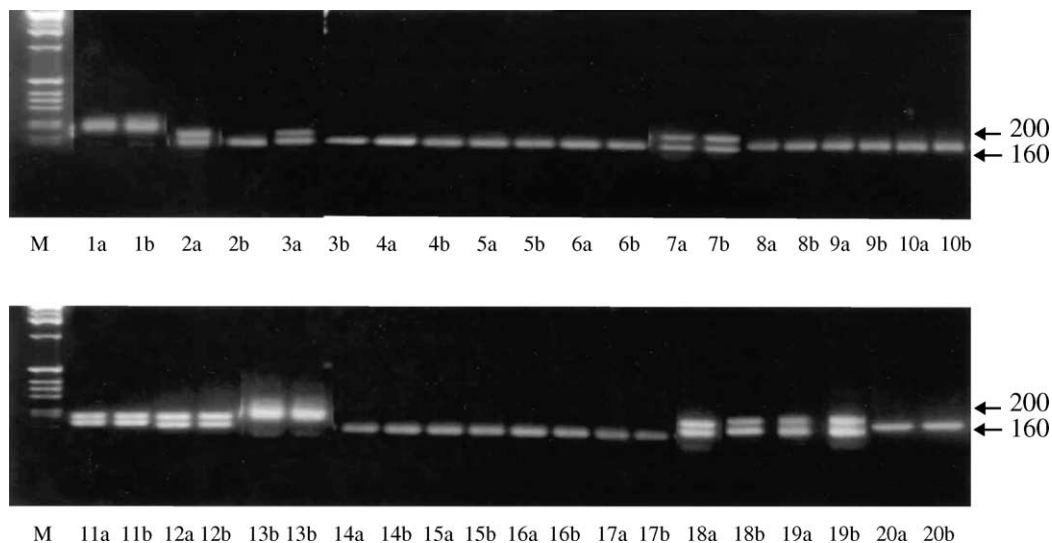


Fig. 1. Incidence of LOH of *DCC* gene in macroscopically normal rectal mucosa at the beginning (baseline, (a) i.e. at the time of polypectomy) and 1 year after treatment (b) with supplemental folic acid or placebo tablets in polypectomized patients. Heterozygous individuals have both 200 and 160 bp bands whereas homozygous individuals show either the 200 or 160 bp band. Patient numbers and treatment groups are given in Table 1. “M” represents molecular weight marker.

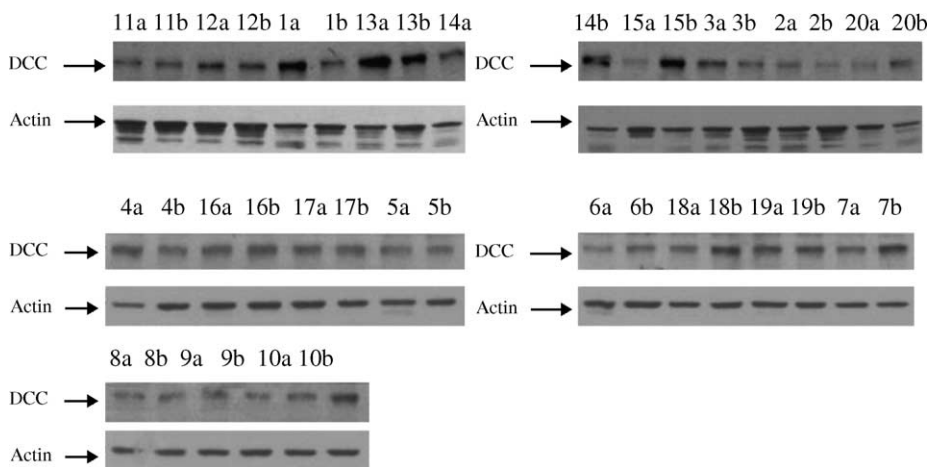


Fig. 2. Western blot showing changes in the levels of DCC protein in macroscopically normal mucosa at (baseline, (a) i.e. at the time of polypectomy) and 1 year after treatment (b) with supplemental folic acid or placebo tablets in polypectomized patients. Twenty micrograms of total mucosal protein from each subject were analyzed. At the completion of DCC protein analyses, membranes were stripped and subsequently probed with β-actin antibodies. Relative change in DCC protein level in each subject was expressed as a DCC/β-actin ratio as shown in Fig. 3.

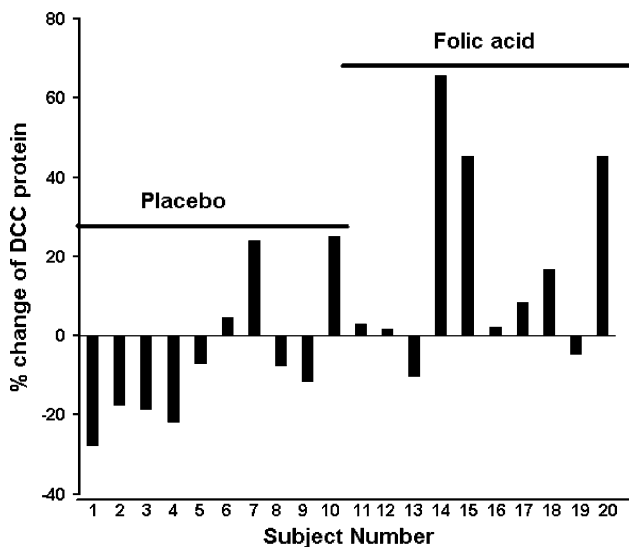


Fig. 3. Relative changes in DCC protein levels, expressed as DCC/β-actin ratios. Values are derived from the data shown in Fig. 2. Protein levels are presented as percent change over the corresponding control.

of PCNA immunoreactive cells per colonic crypt, whereas an 88% ($P < 0.05$) increase was observed in the placebo group, when compared with the corresponding baseline values (Table 4).

Table 4
Changes in PCNA-immunoreactive cells in macroscopically normal rectal mucosa at the beginning (baseline, i.e. at the time of polypectomy) and 1 year after treatment with folic acid or placebo in polypectomized patients

Treatments	PCNA immunoreactive cells per crypt	
	Baseline	1-year
Folic acid ($n = 10$)	6.24 ± 1.04^a	5.27 ± 2.9 (NS)
Placebo ($n = 10$)	5.52 ± 4.18^a	10.40 ± 6.5 ($P < 0.05$)

^a Baseline folate vs. placebo: $P = NS$.

4. Discussion

It has been demonstrated that folate deficiency, a risk factor for colorectal carcinogenesis [3–5], can disrupt DNA methylation [30] and induce misincorporation of uracil for thymidine during DNA synthesis [31] resulting in increased spontaneous mutation [32] as well as chromosomal abnormalities and errors in DNA synthesis [33,34]. The restoration of DNA methylation status in patients with colorectal neoplasms treated with supraphysiological doses of folic acid [35] lends further support to this hypothesis. In addition to restoration of DNA methylation status, folic acid appears to affect other intracellular events that are critically involved in regulating cell proliferation. Earlier studies from this laboratory have demonstrated that supplemental folic

acid markedly inhibits carcinogen-induced stimulation of tyrosine kinase activity in rat colonic explants [36]. Furthermore, we have reported that supplemental folic acid inhibits proliferation as well as the activity and expression of EGFR in human colon cancer cell lines [13]. There is abundant evidence suggesting that EGFR is involved in regulating the development and progression of colorectal cancer [37–40]. Our data suggest, for the first time, that patients with colorectal adenomas may demonstrate a time-dependent increase in mucosal proliferation which is suppressed by folic acid. However, the results should be viewed with caution since the number of subjects in our pilot study was small.

Progression of normal epithelium to carcinoma, however, is a multi-step process resulting from the accumulation of mutations [15,16]. It is becoming increasingly apparent that tumor suppressor genes play a key role in the development and progression of carcinogenesis since the products of these genes normally function to regulate cell growth and differentiation and their loss of function contributes to the neoplastic phenotype [41]. For example, the tumor suppressor gene *p53* is considered to play a crucial role in cellular proliferation and apoptosis and as the guardian of genomic integrity [19,42]. The loss or inactivation of the tumor suppressor gene *APC*, which initiates genomic instability, is thought to produce adenomas in the colon predisposing it to carcinogenesis [16,17]. *DCC*, another tumor suppressor gene present in the long arm of chromosome 18, has been shown to have allelic deletions and/or loss of expression in gastrointestinal and other carcinomas [18,24]. To the best of our knowledge, no information is available as to whether supplemental folic acid might be protective of mutation of tumor suppressor gene(s) in the colorectal mucosa. Supplemental folic acid has been shown to be protective of mutations involving *G* → *T* transversions, which have been related to poor prognosis and a high risk of recurrence in colorectal carcinomas [43]. Our current data, for the first time, show that in polypectomized patients, treatment with supplemental folic, but not placebo prevents LOH of *DCC* gene accompanied by increased expression of DCC protein. Since reduced expression of DCC is thought to play an important role in malignant transformation [29], our current observation of a complete loss of one allele in *DCC* gene in two out of four placebo-treated subjects together with decreased expression of DCC protein in these and other placebo-treated subjects raises the possibility that they may be vulnerable to malignant transformation. Decreased expression of DCC protein together with increased mucosal proliferation in placebo-treated patients lends further credence to the time-related cumulative changes in the propensity for colorectal carcinogenesis, an effect(s) which may be suppressed by folic acid. Although the mechanism(s) by which folic acid exerts this action is not fully understood, it is plausible that supplemental folic acid prevents uracil misincorporation into DNA which leads to increased chromosome breakage, a risk factor for cancer [44,45]. Another possibility could be due to regional differences in the methylation status of the *DCC* gene. For ex-

ample, Sato et al. [46] noted discrepancies between methylation status and DCC expression in primary gastric cancer which may have been related to differences in methylation status between the heart of promoter CpG islands and the region which they examined. It has been suggested that a small methylated region of *hMLH1* could itself block expression [47]. Although we did not specifically explore the methylation status of *hMLH1* gene, we found no differences in the incidence of mutations in *hMLH1* gene between the placebo and folic acid-treated groups (data not shown).

In contrast to what has been observed for *DCC*, no LOH was observed for either *APC* or *p53* gene in any of the subjects. This observation suggests that the induction of colorectal adenoma(s) in these subjects was not the result of LOH of either *APC* or *p53* gene. However, since LOH of *APC* and *p53* was assessed by studying RFLP in exon 11 and codon 72, respectively, the possibility of other areas of these genes being affected in the development of colonic adenomas cannot be totally disregarded.

In conclusion, our results demonstrate increased LOH of *DCC*, but not of *APC* or *p53* gene in macroscopically normal rectal mucosa in patients harboring adenomatous polyps. The LOH of *DCC* gene was also accompanied by a concomitant reduction in the levels its protein. Decreased DCC proteins levels were also observed in some of the individuals who demonstrated no LOH of DCC. Supplementation of folic acid in polypectomized patients resulted in stabilization of DCC allele. Folic acid supplementation was also found to stabilize or increase the levels of DCC protein in macroscopically normal appearing rectal tissues in polypectomized patients.

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