Chemopreventive effect of dietary glutamine on colitis-associated colon tumorigenesis in mice

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Introduction

Colorectal cancer (CRC) is one of the most common forms of fatal cancer in the world, however, the underlying molecular pathogenesis and effective strategies for prevention and treatment of CRC are poorly uncovered (1). Chronic inflammation is a known risk factor for carcinogenesis, indeed, accumulated data indicate that up to 15% of human cancer incidence is associated with inflammation (2). Inflammatory bowel diseases, which mainly included ulcerative colitis (UC) and Crohn’s disease, show a major increased risk for CRC, yet the mechanisms that link these chronic colitis to CRC development are in large part unknown (3). It was reported patients with UC have a significantly higher risk for the development of CRC (4). Inflammation-induced CRC develops in patients with UC is increasing as the duration of disease increases, with a cumulative activity index; DSS, dextran sulfate sodium; GLN, glutamine; IL, interleukin; MPO, myeloperoxidase; NF-κB, nuclear factor-xB; NO, nitric oxide; SD, standard deviation; TNF-α, tumor necrosis factor-α; UC, ulcerative colitis.

Abbreviations: AOM, azoxymethane; CRC, colorectal cancer; DAI, disease activity index; DSS, dextran sulfate sodium; GLN, glutamine; IL, interleukin-6; INOS, inducible nitric oxide synthase; MPO, myeloperoxidase; NF-xB, nuclear factor-xB; NO, nitric oxide; SD, standard deviation; TNF-α, tumor necrosis factor-α; UC, ulcerative colitis.

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Although colonoscopic surveillance in patients with long-standing UC is currently recommended, colitis-associated CRC still accounts for high rate of all deaths in patients with UC (6). The mechanisms underlying the association between colitis and carcinogenesis are largely unknown, and the currently suggested mechanisms include chronic formation of reactive oxygen species and tumorigenesis induced by inflammatory stimuli such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (7). In addition, the importance of inflammation is further highlighted by the dependence of tumor growth and progression on the activation of nuclear factor-xB (NF-xB), which is considered crucial for tumor growth and progression (8). Indeed, data indicated NF-xB pathway activation observed during inflammatory bowel diseases may contribute to tumor formation by providing antiapoptotic survival signals to the colonic epithelial cells (8).

Glutamine (GLN) is a non-essential amino acid that appears to be conditionally essential during periods of physiologic stress. It is a critical substrate for enterocytes as well as rapidly proliferating immune cells (9). Accumulated data showed that GLN has immune-modulating effects and is considered an essential amino acid in catabolic conditions (10). It has been shown that deficiency of GLN results in damage to the gut epithelium (11). Enteral GLN is the main source of amino acids for the enteral mucosa, it has been used in many clinical conditions including trauma, burning and chemotherapy to prevent enteritis and colitis (11). Nutritional support for the gut by GLN supplementation stimulates mucosal cell growth to aid repair of mucosal injury and improves intestinal structure and function as well as human disorders (12). It has been found that GLN supplementation has no clear benefit both in patients with inflammatory bowel diseases (13) and in animals receiving 2,4,6-trinitrobenzene sulfonic acid (14), and there is also a report indicated that GLN exacerbates colitis in rats (15). However, most studies support a protective effect of GLN administration in experimental colitis, as administration of GLN reduces colonic damage in experimental colitis (16,17). Vicario et al. (18) also found that GLN supplementation improved barrier functions in rats with dextran sulfate sodium (DSS)-induced colitis. It was also reported that oral feeding with GLN showed protective effect on experimental colitis (19), and dietary GLN improves mucosal function in experimental colitis (18). The mechanisms of GLN attenuation of colitis mainly included prevention of NF-xB activation (20) and suppression of Th1/Th17/Th2 cytokine production (21). Although it is recognized that GLN is rapidly absorbed in the proximal small intestine and may not reach the inflamed part of the intestine at a sufficient concentration to achieve its anti-inflammatory effects (22), oral GLN still showed its benefit effect on colon tissue (19). As it was accepted that GLN shows therapeutic activity in colitis, however, few studies have been performed focusing on GLN in colitis-associated CRC till now. Clearly, additional studies are needed to explore the chemopreventive activity of GLN in antigenetic effects.

The aim of this study was to evaluate the ability of oral GLN to inhibit colitis-associated colorectal neoplasia. The DSS/azoxymethane (AOM) model of induced colitis-associated CRC was selected for these chemopreventive analyses based on the induction of dysplastic lesions. Our resulting data strongly suggest that long-term oral-receiving GLN reduces susceptibility for colitis-associated colorectal carcinogenesis. GLN administered orally markedly decreased the severity of macroscopic damage and reduced the tumor burden of animals with DSS/AOM-induced colitis-associated CRC.

Materials and methods

Animal treatment

The DSS/AOM model of colitis-associated colorectal carcinogenesis was employed in this study according to the experimental treatment protocol...
reported previously (23,24). Female BALB/c mice (6 weeks of age) were purchased from Laboratory Animal Center of Nanjing Medical University (Nanjing, China) and maintained in plastic cages with stainless-steel grid tops under laboratory conditions (18–25°C temperature, 60–70% humidity and natural light). BALB/c mice were selected in this study as it was reported that the incidence of colonic tumorigenesis in the DSS/AOM-challenged BALB/c strain was 100% (25). All mice had free access to diet and water, and before starting the experiments, they experienced an acclimation period of 1 week. All experimental manipulations were undertaken in accordance with the institutional guidelines for the care and use of laboratory animals and the study was approved by ethics committee of Nanjing Medical University.

There were one control group (n = 20) and two DSS/AOM groups (n = 20 for each group) in this study. Mice in DSS/AOM groups were injected intraperitoneally with a single dose of the mutagenic agent AOM (7.4 mg/kg) (Sigma Chemical Co., St Louis, MO) followed by three cycles of 3% DSS (International Laboratory (IL), South San Francisco, CA) in drinking water for 1 week and normal drinking water for 2 weeks (Figure 1A) (24). One of the DSS/AOM groups was administered with oral GLN-enriched diet (3 g/100 kcal, GLN group, GLN was obtained from Sigma–Aldrich), whereas mice in the other DSS/AOM group (model group) and control group received a control diet (26,27). Mice in the GLN group were given 8.5 g GLN/kg/day orally, and the diets for mice were isonitrogenous and isocaloric (Table I). The diets were given in form of pellets. All mice in the GLN group received GLN-enriched diet for 1 week at the beginning of the experiment and then were administrated with GLN-enriched diet every other week, while a control diet was given to mice in GLN group during the period when GLN-enriched diet was stopped (Figure 1A). Intermittent treatment of long duration was chosen as it was reported that intermittent administration may not impair renal function (28).

Body weights, food and drink consumptions were monitored once per week throughout the experiment. Clinical assessment of all DSS/AOM-treated animals for body weight, stool consistency, rectal bleeding and general appearance was performed weekly. Stool probes were tested to evaluate rectal bleeding by using Hemoccult paper (Beckman Coulter, Fullerton, CA). At day 80 of the experiment, all mice were killed, and the whole colorectal tissues were collected for chemical and pathological analyses.

**Evaluation of the severity of clinical colitis**

At weekly intervals, the disease activity index (DAI) of mice in model group and GLN group was observed in each mouse by using the numerical system described by Cooper et al. (29). The score was ranged from 0 to 4 and was the sum of scores of body weight loss (0 = none; 1 = 1–5%; 2 = 5–10%; 3 = 10–20%; 4 = over 20%), stool consistency (0 = well-formed pellets; 2 = loose stools; 4 = diarrhea) and presence or absence of fecal blood (0 = negative hemoccult test; 2 = positive hemoccult test; 4 = gross bleeding).

**Macroscopic and histopathological evaluation analysis**

Mice were killed under anesthesia at day 80. The colon was excised from the ileocecal junction to the anal verge, rinsed with phosphate-buffered saline and plasma was collected. Colon length (from the colocele junction to the rectum) was measured for assessment of morphologic change. The entire colon tissues were prepared for histological analysis and further examined.

The colon was opened longitudinally. Gross examination was performed to measure the pattern of tumor development, including quantity, and location of each tumor within the large bowel. The tumor incidence was defined as number of mice with tumors/total mice in the group, whereas the tumor burden was estimated histopathologically by squaring the mean diameter and multiplying by the number of lesions (adenoma and adenocarcinoma) evaluated per mouse. Furthermore, the size of each identified microscopic lesion was determined by measuring the largest diameter of the lesion using an ocular micrometer.

After collection and flushing with phosphate-buffered saline, tissue samples were fixed immediately in 10% formalin for 24 h and then paraffin-embedded. Six micrometer sections were stained with hematoxylin and eosin for light microscopic examination. Sample sections of the colon were photographed with a Zeiss Axioplan (Zeiss, Thornwood, NY) photomicroscope. All sections were randomized and evaluated by a trained observer who was blinded to the treatment groups. The degree of inflammation within the entire colon and rectum was quantified histopathologically, as described previously (29,30). Briefly, the severity of inflammation was graded on a scale from 0 to 3 (0 = no inflammation, 1 = mild, 2 = moderate and 3 = severe), and the thickness of inflammatory involvement was ranged from 0 to 3 (0 = no inflammation, 1 = mucosa, 2 = mucosa plus submucosa and 3 = transmural). Additionally, the severity of epithelial damage was evaluated from 0 to 3 (0 = intact epithelium, 1 = mild, 2 = moderate and 3 = severe).

**Fig. 1.** Experimental protocol, DAI and colon inflammation for establishment of inflammation-related mouse colon carcinogenesis model with or without GLN-enriched diet. (A) Experimental protocol for inducing inflammation-related mouse colon carcinogenesis model (GLN means GLN-enriched diet and C means control diet). (B) DAI was significantly decreased when inflammation-related carcinogenesis mice received GLN (*P < 0.05). Colon length (C) as well as colon weight (D) in mouse model of colitis-associated cancer were significantly improved in animals receiving GLN-enriched diet (*P < 0.05).
Table I. Composition of diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control</th>
<th>GLN-enriched diet</th>
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<tbody>
<tr>
<td>Whey protein (g)</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Carbohydrate (g)</td>
<td>16</td>
<td>16</td>
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<tr>
<td>Fat (g)</td>
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<tr>
<td>Mineral (g)</td>
<td>1.91</td>
<td>1.91</td>
</tr>
<tr>
<td>Cellulose (g)</td>
<td>0.76</td>
<td>0.76</td>
</tr>
<tr>
<td>Vitamin (g)</td>
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<td>0.38</td>
</tr>
<tr>
<td>Glutamine (g)</td>
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<td></td>
</tr>
<tr>
<td>Glycine (g)</td>
<td>3.52</td>
<td>0</td>
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1 = disruption of architectural structure, 2 = erosion and 3 = ulceration) and extent of lesions was also calculated (0 = no lesions, 1 = punctuate, 2 = multifocal and 3 = diffuse). Sections stained with hematoxylin and eosin were also histopathologically evaluated for neoplastic lesions in a blinded manner as described previously (25, 31).

Enzyme-linked immunosorbent assay

Colon tissues were washed with 1x PBS (pH 7.5) to remove fecal contents. For the levels of TNF-α, IL-6, myeloperoxidase (MPO) and eicosanoids (PGE2 and LTB4) evaluation, tissue samples were powdered, weighed and homogenized in lysis buffer. Homogenized tissue samples were centrifuged at 1000g for 15 min at 4°C. Supernatant was again centrifuged at 1000g for 15 min and was collected. TNF-α, IL-6 and MPO concentrations were measured using specific Enzyme-Linked Immunosorbent Assay Kits for TNF-α (Biosource International, Inc., Camarillo, CA), IL-6 (R&D Systems, Minneapolis, MN), MPO (Cell Sciences, Canton, MA) and for eicosanoids (PGE2 and LTB4) (R&D Systems) according to the manufacturer’s protocol.

In addition, transAM NF-κB p65 Activation Assay (Active Motif, Carlsbad, CA) was performed to evaluate the activated p65/RelA protein. After collection of cell extracts from frozen colon tissues by using Nuclear Extract Kit (Active Motif), the protein concentrations were then determined. As described by Nowak et al. (30), lysates were incubated at room temperature for 1 h in dishes containing immobilized oligonucleotides that comprise the NF-κB consensus DNA-binding site (5′-GGGACTTTCC-3′). The activated p65/RelA protein concentration was then evaluated as per the manufacturer’s recommendation.

Quantitative real-time PCR

Total RNA was extracted from colon tissues and complementary DNA was synthesized. Primer sequences used for complementary DNA amplification were as follows: β-actin: 5′-GACGCCGCGTGCTACATCCTG-3′ and 5′-AAGGAGCCTGCGAAGGACCC-3′; IL-1β: 5′-GTTGACTGTGGAGGAAGCTGTG-3′ and 5′-GAAGGTTCCAGGGGAAAAGAC-3′; IL-6: 5′-GATGGAAACAGGATGTACATT-3′ and 5′-ATGGTACCTGAGAACCACAGAGGA-3′; interferon-γ: 5′-CTCTGGCGCTGATGCTGCTG-3′ and 5′-TCCGCTTTGCTGGTCCTCTG-3′; TNF-α: 5′-GAACTGCGAGAGAGGGCTATCCT-3′ and 5′-GGTCTCTACCCTGAGAACTGTA-3′; CCL2: 5′-GCCTGCTATTGACCCATCTTG-3′ and 5′-TTTGGATCTGAACTGGAATG-3′; CCL3: 5′-CTGCTGCTGTTGCTGTCCTG-3′ and 5′-TGGCTACCCTGCTGCTGTCCTG-3′; CCL4: 5′-TCACCGCTGCTGCTGCTGCTGTCCTG-3′ and 5′-TGGCTACCCTGCTGCTGCTGTCCTG-3′; CCL11: 5′-GGCTCTGGTTACCTGCTGCTGTCCTG-3′ and 5′-GGCTCTGGTTACCTGCTGCTGTCCTG-3′; iNOS: 5′-GGAAGGTCCACGGGAAAGACAC-3′ and 5′-GGTGCTGAAGACCTTAGGGCAGAT-3′; COX-2: 5′-GCTCACCCCTCTGCTGCTGCTGCTGTCCTG-3′ and 5′-GCTCACCCCTCTGCTGCTGCTGCTGTCCTG-3′; CCL3: 5′-CCCTTTGCTCTTACCTCCTTCTCCT-3′ and 5′-CCCTTTGCTCTTACCTCCTTCTCCT-3′.

Fast Real-Time PCR System (7300; Applied Biosystems, Foster City, CA) was used for thermal cycling and real-time fluorescence measurements. The quantitative real-time PCR with SYBR green PCR Master Mix (Applied Biosystems) was performed in a MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems). All PCR reactions were all triplicate with the product amplification results normalized to β-actin expression for each sample.

Western blot analysis for cyclooxygenase-2 and inducible nitric oxide synthase

As described previously (24), colon tissues were washed with Hank’s balanced salt solution. After washing, the tissue was homogenized in a lysis buffer and a tablet of protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The extracts of the tissue were separated on a 10% or 12% denaturing polyacrylamide gel and transferred to a polyvinylidene difluoride membrane by electrophotting. Transferred membranes were then blocked for 2 h at room temperature with 5% non-fat dried milk in 1x Tris-buffered saline and 0.1% Tween-20 before incubating with antibodies against cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) or β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 dilution in blocking buffer overnight at 4°C. After washing with Tris-buffered saline and 0.1% Tween-20, the membranes were incubated with a peroxidase-conjugated secondary antibody, diluted at 1:10 000 in blocking buffer, for 1 h at room temperature. Detection was performed by incubating the membranes with enhanced chemiluminescence plus (AMRESCO) and being exposed to X-ray films.

Nitric oxide production assays

For detection of the nitric oxide (NO) production, colon tissues were placed in lysis buffer, and the NO analysis was performed as described by Soyturk et al. (32). Briefly, the colon tissue lysates were mixed with an equal volume of Griess reagent (with 0.1% naphthalene diamine dihydrochloride and 1% sulfuramidine in orthophosphoric acid) in a 96-well microtitr plate and then incubated for 10 min at room temperature. The absorbance was measured at 540nm. The amount of nitrite released was quantified by comparison with sodium nitrate as standard. No concentration was expressed as nanomolar per milligram tissue.

Immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

For immunohistochemistry analysis of proliferation, sections were incubated with antibodies against Ki-67 (Dako, Carpinteria, CA) overnight at 4°C, which was followed by biotinylated secondary antibodies (Vector Labs, Burlingame, CA) and streptavidin–horseradish peroxidase plus diaminobenzidine (Vector Labs, Burlingame, CA). To detect the apoptosis, section of tissue was deparaffinized and dehydrated through washes with graded concentrations of ethanol. Apoptotic cells were detected using ApoAlert DNA Fragmentation Assay Kit (BD Biosciences, Palo Alto, CA) as described previously (24). The number of apoptotic or proliferating cells per crypt was determined as it was reported (33).

Statistical analyses

All values are presented as mean ± standard deviation (SD). Statistical significance of evaluated data was tested by using Mann–Whitney U-test, Student’s t-test, two-way analysis of variance or Kruskal–Wallis test. Chi-square test was used for comparison of the descriptive values. P < 0.05 was considered to be significant.

Results

DSS/AOM-induced colorectal inflammation

All mice in the DSS/AOM-treated groups survived. The administration of GLN resulted in an attenuation of signs of colitis as established by DAI, the colon length, colon weight and microscopic features of colon. The individual DAI, which was used to monitor the therapeutic benefit of treatment, was assigned once a week after the experiment proceeded. As shown in Figure 1B, mice in GLN group showed lower mean DAI values (except w3 and w6) when compared with mice of model group. It was reported that colorectal inflammation has been correlated with decreased colon length in rodent models of colitis (34,35), and the length of colon is widely accepted as a reliable inflammation index of colitis (36). Our results indicated that GLN-treated mice had significantly longer colons (Figure 1C) and improved colon weight (Figure 1D) than colitis-associated CRC model mice. Furthermore, our results clearly indicated the microscopic evaluation of the colitis was dramatically improved in mice with GLN-enriched diet when compared with standard diet after treatment with DSS/AOM, which was documented by decreased in inflammation severity, damage, mucosal thickness and inflammation extent in colons of mice in GLN group (Figure 2A). Thus, GLN had therapeutic activity in mice with DSS/AOM-induced colitis.

Pro-inflammatory cytokine levels, chemokine expression and MPO concentration in colitis-associated colorectal carcinogenesis

To investigate the effects of GLN-enriched diet on the expression of pro-inflammatory cytokines and MPO, enzyme-linked immunosorbent assay was used to analyze the production of TNF-α, MPO and IL-6 in the colonic tissues. The production of TNF-α (Figure 2B) and IL-6 (Figure 2D) and the concentration of MPO (Figure 2C) were significantly diminished (P < 0.05) in DSS/AOM-induced mice fed with the GLN-enriched diet compared with those received standard diet. In addition, GLN dramatically decreased chemokine and cytokine gene expression in DSS/AOM mice with colitis-associated cancer (Figure 3).
The incidence of colitis-associated neoplastic lesions was analyzed as it was indicated in Figure 4. The dysplastic lesions were tubular adenoma or adenocarcinoma histopathologically (Figure 4A). Results indicated colonic neoplasms developed with a different incidence and multiplicity for each group of mice that treated with DSS/AOM. The incidence of colonic neoplasms in the DSS/AOM-induced mice (100%) was higher than those received GLN-enriched diet (65%, \( P = 0.0036 \); Figure 4B). Our results were in a line with Suzuki et al. (25), who reported that all BALB/c mice that received DSS/AOM developed colonic neoplasms. In addition, the incidence of colonic adenoma in DSS/AOM-induced colitis-associated CRC model mice was 80%. On the contrary, DSS/AOM-induced animals fed with the GLN-enriched diet had lower incidence (60%, \( P = 0.168 \)) although it did not reach the statistical difference (Figure 4B). Our data also showed the incidence of colonic adenocarcinoma was 100% in the mice treated with DSS/AOM. Nevertheless, the incidence of adenocarcinomas was reduced significantly in CLN-fed mice (60%, \( P = 0.035 \); Figure 4B). The number, size and location of detectable tumors were also examined in this study. As the data indicated, there were significantly fewer tumors in the colitis-associated CRC model mice than in those given CLN-enriched diet (\( P < 0.05 \); Figure 4C). Tumors in the intermediate part of colons of mice in GLN group were smaller than those in model group (\( P < 0.05 \); Figure 4D).
COX-2 and iNOS expression and NF-κB activity
Results from our data clearly indicated that exposure to DSS/AOM caused strong expression of COX-2 (P < 0.05; Figure 5A and D) and iNOS (P < 0.05; Figure 5B and E) in cytosolic extracts from colonic tissue. Interestingly, these effects were abolished by oral administration of GLN, which significantly induced downregulation of COX-2 (P < 0.05; Figure 5A and D) and iNOS (P < 0.05; Figure 5B and E) in DSS/AOM-treated mice. NF-κB activity was also assessed by measuring activated p65 in cell extracts from the colons of mice in all three groups. There was significantly higher activity of NF-κB in the DSS/AOM-induced colitis-associated CRC mice, as compared with the control group (Figure 5C). In contrast, the activity of NF-κB was dramatically decreased in those GLN-fed mice (P < 0.05; Figure 5C). Furthermore, our data indicated that eicosanoids production (PGE2 and LTB4) and NO level were significantly decreased in colon tissues of DSS/AOM-treated mice receiving GLN-enriched diet (P < 0.05; Figure 6).

Effect of GLN on cell proliferation and apoptosis in vivo
To explore the effect of GLN treatment on proliferation and apoptosis in inflammation-related CRC mice model, immunohistochemistry studies with Ki-67, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay and c-myc gene expression were performed. The results showed increased apoptosis and decreased proliferation in mice receiving GLN-enriched diet as compared with those receiving control diet (Figure 7). This was confirmed by the reduced expression of c-myc messenger RNA (Figure 7).

Discussion
The role of interactions between genetic, immunologic, microbial and environmental factors is expected in UC, but exact pathogenesis still remains unclear. Long-standing UC has an increased risk to develop CRC. The risk of CRC development is much higher in patients with UC than in the general population (37). The tumor-promoting effect of inflammation is now widely recognized by inducing gene mutations and epigenetic alterations and by increasing the expression of factors involved in carcinogenesis (such as NF-κB and COX-2), releasing of reactive oxygen, inhibiting apoptosis or stimulating cell proliferation. It was also widely accepted that chronic inflammation promotes carcinogenesis by inducing production of a variety of cytokines and chemokines that propagate a localized inflammatory response by activating transcription factors such as NF-κB (38), which is accompanied by increased transcription levels of inducible forms of COX-2, iNOS and pro-inflammatory cytokines including TNF-α and IL-6 (39). These findings have important implications for the development of anticancer therapies and offer an opportunity to devise strategies to develop anti-inflammatory drugs for cancer therapy.

A number of animal models mimicking different aspects of UC have been developed. In mice, an experimental colitis is probably the most commonly induced by oral administration of DSS (40). DSS induces acute colitis with symptomatic and histopathologic findings similar to UC. Induction and maintenance of UC might be one of the primary therapeutic goals in order to decrease the likelihood of CRC developing. The focus of this study was to explore the potential protective role of GLN administration by diet in the development of carcinogenesis from a DSS/AOM-induced CRC mouse model. GLN, although mainly a metabolic fuel for the small bowel cells (41), is also used increasingly as a fuel by the colonocytes and showed benefit effects in colonic disorders (19,42,43). It was reported that butyrate uptake by colonocytes involves the monocarboxylate transporter (MCT) 1 and butyrate oxidation deficiency in intestinal inflammation is the consequence of reduction in monocarboxylate transporter-1-mediated butyrate uptake (44). Whether GLN with the ability to modify glucose metabolism or monocarboxylate transporter-1 transporter expression is uncovered, further studies may focus on this topic and explore the involved mechanisms. GLN has been used clinically in patients with extended trauma, enteritis and colitis (11). It was also reported that GLN significantly reduced gross damage and histopathological scores in the animals receiving acetic acid (20). Additionally, GLN-enriched elemental diets have been shown to reduce portal endotoxin level in experimental colitis (45). Earlier studies have demonstrated that GLN also had a positive impact on the intestinal barrier by reducing permeability and bacterial

Fig. 4. Effect of GLN-enriched diet on the tumor incidence and burden of colonic tumorigenesis. (A) Histopathology of colonic neoplasms developed in mice of model group (a and b) and GLN group (c and d). (a and c) ×100 and (b and d) ×200. (B) Tumor incidence of UC-associated CRC in animals treated with DSS/AOM and fed with GLN-enriched diet or standard diet. (C) Multiplicity of colonic tumors in animals treated with DSS/AOM and fed with GLN-enriched diet or standard diet. (D) Comparison of tumor size in mice of model group. Data are expressed as the means ± SD, *P < 0.05.
translocation to physiologic levels and preserving mucosal integrity (46). Furthermore, oral GLN supply seems to be a suitable approach for improving intestinal immunity in immunocompromised status (27). Currently, it was recognized that the anti-inflammatory activity of GLN in colitis may be mediated, at least in part, by inhibition of the expression of certain pro-inflammatory mediators (20). Although it was recognized that GLN has effective activity in UC-like colitis, studies focusing on this agent in UC-associated CRC have been rarely reported. Therefore, it is interesting to see how our results show a preventive/protective role for dietary GLN in the DSS/AOM-induced colitis-associated CRC animal model.

MPO is an useful indicator of the extent of neutrophil infiltration, which could enhance the growth and survival of premalignant cells. Our results indicated the decreased concentration of MPO in colon tissue of DSS/AOM-treated mice; in contrast, this was reversed by GLN-enriched diet. CRC arises as a result of sequential episodes of activating pro-inflammatory cytokines such as TNF-α and IL-6. Our study shows that cytokines and chemokine expression were increased significantly in response to DSS/AOM treatment, which may be involved in ‘inflammation–carcinoma sequence.’ On the contrary, the GLN diet decreased the cytokines and chemokine expression significantly, which was in a line with the data that indicated oral feeding with GLN modulates and attenuates the colonic inflammation in animal colitis (19,42). These results suggested that GLN appears to be able to exert anti-inflammatory and growth inhibitory effects on CRC development by reducing cytokines and chemokine expression.

Our results demonstrate that GLN, the main source of amino acids for the enteral mucosa agent, given as a dietary supplement, clearly shows antitumor activity. The mice treated with DSS/AOM that received GLN-enriched diet presented a minor incidence of colitis-associated colonic neoplasms. This positive effect is well related with the decreased proinflammatory cytokines, attenuated colorectal inflammation and a lack of COX-2 and iNOS expression in the colonic tissue at the end of the experimental model. Moreover, a reduced NF-κB activity, which was considered as the lynchpin for inflammation-associated cancer (47), was also observed. Reduced NF-κB activity and decreased expression of iNOS and COX-2 confirmed the anti-inflammatory effect of GLN. Furthermore, this anti-inflammation effect was confirmed by the results that eicosanoids production and NO level were significantly decreased in colon tissues of mice receiving GLN-enriched diet. These results suggested the preventive effect of GLN in colitis-associated CRC and implied the potential usefulness of this common used element of nutrition as a chemopreventive agent for individuals such as patients with UC who presented high risk for CRC development. The effects of GLN on cell proliferation and apoptosis in vivo were also evaluated. GLN indicated induced effect on apoptosis and reduced activity on proliferation in colon tissue of mice treated with DSS/AOM. Our results suggested
Antitumor effects of glutamine

The results presented in this study show that a GLN-enriched diet decreases inflammation-triggered colon tumorigenesis. The suppressed tumorigenesis in the GLN-feeding DSS/AOM-treated mouse was evidenced by a lower incidence of colon neoplasia as well as a smaller size of the tumors formed. The lower degree of inflammatory changes was associated with a decreased tumor incidence in the colon. These changes were, on a molecular level, accompanied by a lower expression of iNOS and COX-2 in colonic tissue. The overexpression of COX-2 and iNOS is considered associated with colon tumor promotion by a number of potential mechanisms (48), as COX-2 can promote cell growth, angiogenesis and suppression of immunity, and iNOS can induce the expression of NO, which is able to cause DNA damage (49). Recent data have implicated pro-inflammatory iNOS expression and nitrosative stress in the growth of colon carcinomas by nitrosylation of caspases and subsequent inhibition of apoptosis, whereas strategies to lower NO have been tested experimentally for the prevention of colon tumorigenesis (50). Indeed, both of these protein expressions significantly increased in mice receiving DSS/AOM as indicated in our results. However, the GLN diet reduced significantly both COX-2 and iNOS expression versus standard diet. The data presented in this study are consistent with previous findings, which indicate that GLN can inhibit the expression of pro-inflammatory mediators and suppress nitrosative stress in colon (46,51). The observed inhibition of colon tumor formation and growth rate by the GLN-enriched diet is probably due to the inhibitory effect of these fatty acids on NF-κB, and thus suppression of NF-κB-mediated pro-inflammatory, pro-proliferative and antiapoptotic activities (46,51). These results are consistent with the previous studies where anti-inflammatory activity of GLN in a colitis model may be mediated by inhibition of NF-κB signaling pathway (20). Leclère et al. (52) reported that GLN was able to decrease NF-κB p65 subunit expression in colonic biopsies from Crohn’s patients. The mechanisms of action of GLN on NF-κB could also involve the regulation of proteolysis of IκBα as described previously by Hubert-Buron et al. (53).

Together with the suppressed NF-κB activity observed in this study, the results showing a decrease of tumor incidence in the colons and a decreased tumor size in GLN group support the link between inflammation and tumorigenesis through increased NF-κB activity in the inflamed colon.

In summary, the ability of GLN to prevent the development of the colonic carcinogenesis was demonstrated by a better DAI index, an attenuated colorectal inflammation and lower tumor incidence of DSS/AOM-treated mice. We suggested in this study that the chemopreventive effect of GLN for colitis-associated carcinogenesis could be mediated mainly through the anti-inflammatory mechanisms, such as reduction of the pro-inflammatory cytokine levels, decrease of COX-2 and iNOS protein overexpression and inhibition of NF-κB activity in the colonic tissue.

Funding
Nanjing Medical University (No. 2011NJMU245); Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Acknowledgements
The authors would like to thank Dr Yi Li (Jinling Hospital, Medical School of Nanjing University) for the excellent pathological technical support.

Conflict of Interest Statement: None declared.

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