n-3 Polyunsaturated fatty acids modulate carcinogen-directed non-coding microRNA signatures in rat colon

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We have hypothesized that dietary modulation of intestinal non-coding RNA [microRNA (miRNA)] expression may contribute to the chemoprotective effects of nutritional bioactives (fish oil and pectin). To fully understand the effects of these agents on the expression of miRNAs, Sprague-Dawley rats were fed diets containing corn oil or fish oil with pectin or cellulose and injected with azoxymethane (AOM, a colon-specific carcinogen) or saline (control). Real-time polymerase chain reaction using miRNA-specific primers and Taq ManTM probes was carried out to quantify effects on miRNA expression in colonic mucosa. From 368 mature miRNAs assayed, at an early stage of cancer progression (10 week post AOM injection), let-7d, miR-15b, miR-107, miR-191 and miR-324-5p were significantly (P < 0.05) affected by diet × carcinogen interactions. Overall, fish oil fed animals exhibited the smallest number of differentially expressed miRNAs (AOM versus saline treatment). With respect to the tumor stage (34 week post AOM injection), 46 miRNAs were dysregulated in adenocarcinomas compared with normal mucosa from saline-injected animals. Of the 27 miRNAs expressed at higher (P < 0.05) levels in tumors, miR-34a, 132, 224 and 237 were overexpressed at >10-fold. In contrast, the expression levels of miR-192, 194, 215 and 375 were dramatically reduced (≤0.32-fold) in adenocarcinomas. These results demonstrate for the first time the utility of the rat AOM model and the novel role of fish oil in protecting the colon from carcinogen-induced miRNA dysregulation.

Introduction

With respect to epigenetic mechanisms involved in colon tumor development, it is believed that non-coding microRNAs (miRNAs) control the expression of approximately one-third of the mammalian messenger RNAs (mRNAs) (1). miRNAs act through partial complementation to 3′ untranslated regions of their target mRNAs and regulate mRNA degradation and translation, resulting in inhibition of gene expression in mammals (1,2). Although factors that control the expression of miRNAs are largely unknown, the altered expression of a number of non-coding RNAs have been linked to the development and prognosis of colorectal neoplasia (3–7). Indeed, it is probably that the colorectal ‘miRNAome’ consists of a much larger number of miRNAs than previously appreciated (8). In addition, cellular phenotypes such as apoptosis are regulated by miRNAs and in some cases, upstream and downstream genes have been linked to the epigenetic silencing of miRNAs (9–11). These data suggest that miRNA expression profiles could contribute to a more precise colorectal tumor classification and predict chemotherapeutic outcomes (12). Although it is now possible to analyze portions of the miRNAome using microarray or high throughput polymerase chain reaction (PCR) methodologies (13), to date, the effect of dietary chemopreventive agents on miRNA expression during different stages of colon cancer development has not been determined.

Colorectal cancer continues to pose a serious health problem in the USA. It is estimated that >108 000 new cases and 50 000 deaths occur on an annual basis in the USA (14). From a dietary perspective, a growing number of clinical and experimental studies indicate a protective effect of dietary fish oil, containing n-3 polyunsaturated fatty acids (PUFA), with respect to colon cancer risk (15–24). Eicosapentaenoic acid (20:5n-3,8,11,14,17) and docosahexaenoic acid (22:6n-3,7,10,13,16,19) are typical n-3 PUFA (found in fish oil), defined according to the position of the first double bond from the methyl end of the molecule, which is designated ‘n-3’. In contrast, dietary lipids rich in n-6 PUFA (found in vegetable oils), e.g. linoleic acid (18:2n-6,12) and arachidonic acid (20:4n-6,8,11,14), enhance the development of colon tumors (15,25). These effects are exerted at both the initiation and post-initiation stages of carcinogenesis (15,26). We have also recently demonstrated that the proapoptotic/chemoprotective effect of n-3 PUFA is enhanced when a highly fermentable fiber, pectin (or its fermentation product, butyrate) is added to the diet (26–28). Despite evidence indicating that dietary fish oil and fermentable fiber suppress colon cancer development, we still lack information regarding the precise molecular mechanisms by which docosahexaenoic acid and butyrate protect against colon tumorigenesis.

In the absence of comprehensive human data, the azoxymethane (AOM) chemical carcinogenesis model serves as one of the most definitive means of assessing human colon cancer risk (29,30). We have previously demonstrated that at 10 week post AOM injection, the colonic mucosa is precancerous, e.g. high multiplicity aberrant crypt foci are apparent. Macroscopic tumors are not detectable until ~34 weeks post AOM injection (21). Therefore, to determine the effects of cancer progression on miRNA expression in the colon, we examined AOM-injected rats at both 10 and 34 weeks post initiation. We also tested the hypothesis that an n-3 PUFA-enriched chemoprotective diet will modulate miRNA signatures in the colon.

Materials and methods

Animals

Fifty-four weanling male Sprague-Dawley rats (Harlan, Houston, TX) were acclimated for 2 weeks in a temperature and humidity controlled facility on a 12 h light–dark cycle. The animal use protocol was approved by the University Animal Care Committee of Texas A&M University. The study was a 2 × 2 factorial design with two types of dietary fat (n-6 PUFA or n-3 PUFA), two types of dietary fiber (cellulose or pectin) and two treatments (injection with the colon carcinogen, AOM, or with saline). Animals (n = 6 per group) were stratified by body weight after the acclimation period so that mean initial body weights were not different between groups. Body weight was monitored throughout the study.

Diets

After a 1 week acclimation on standard pelleted diet, rats were assigned to one of four diet groups, which differed in the type of fat and fiber as described previously (26). Diets contained (grams/100 gram diet): dextrose, 51.00; casein, 22.40; d,l-methionine, 0.34; AIN-76 salt mix, 3.91; AIN-76 vitamin mix, 1.12; choline chloride, 0.13 and pectin or cellulose, 6.00. The total fat content of each diet was 15% by weight with the n-6 PUFA diet containing 15.0 g corn oil/100 g diet and the n-3 PUFA diet containing 11.5 g fish oil/100 g diet plus 3.5 g corn oil/100 g diet to prevent essential fatty acid deficiency.

Carcinogen treatment

After 2 week on the experimental diets, six rats per diet group were injected with AOM (Sigma, St Louis, MO) subcutaneous at 15 mg/kg body wt or with...
saline (control). Rats received a second AOM or saline injection 1 week later and were terminated 10 week after the first injection. For generation of colonic tumors, a second group of rats were continued on diet up to 34 weeks post-injection. A section of tumor was taken for RNA isolation as described below and the remainder was fixed in 4% paraformaldehyde and submitted to a histologist for tumor typing (26). All tumors used for RNA analysis were classified as adenocarcinomas by a board-certified pathologist.

**RNA isolation**

Upon termination, each colon was cut open longitudinally, flushed clean with phosphate-buffered saline and 1 cm from the distal colon was collected for miRNA isolation. Epithelial cells were scraped from the underlying muscle layer with a glass microscope slide, homogenized on ice in lysis buffer (mirVana miRNA Isolation Kit, Ambion, Austin, TX) and frozen at −80°C until RNA was isolated. Using the mirVana kit, total RNA enriched with miRNA was isolated following by DNase treatment. Samples were analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to assess RNA integrity and subsequently quantified using a NanoDrop 1000 spectrophotometer (Nanodrop, Wilmington, DE).

**miRNA quantification**

Expression of 368 mature miRNAs was determined using TaqMan Human MicroRNA Panel Low-Density Arrays. As per manufacturer’s instructions, 50 ng RNA was reverse transcribed using the TaqMan MicroRNA RT kit, followed by PCR using Multiplex RT target-specific stem-loop primers (Applied Biosystems, Foster City, CA). Complementary DNA products were diluted, mixed with TaqMan master mix and loaded onto miRNA TaqMan Low-Density Arrays for amplification on an AB 7900HT Real-Time PCR machine. miRNA expression was normalized to 18S rRNA expression.

**Identification of miRNA established targets**

Empirically established miRNA targets were identified using miRecords (http://mirrecords.umn.edu/miRecords), an integrated resource for miRNA-target interactions (31).

**Cell culture**

HCT-116 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum and 2 mM GlutaMAX (Gibco, Carlsbad, CA) at 37°C in 5% CO2. Cells were plated at 1–2 × 106 cells and diluted, mixed with TaqMan master mix and loaded onto miRNA TaqMan Low-Density Arrays for amplification on an AB 7900HT Real-Time PCR machine. miRNA expression was normalized to 18S rRNA expression.

Table I. List of miRNAs expressed in normal rat colon

<table>
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<th>Expression ratio tumor/ control</th>
<th>P-value</th>
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<td>3.07E-02</td>
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<td>miR-99b</td>
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Expression of miRNAs was quantified by reverse transcription using miRNA-specific primers followed by real-time PCR using TaqMan low-density arrays. miRNA expression was normalized to 18S rRNA expression.

miRNA analysis

Twenty-four hours after transfection, cells were harvested and total RNA isolated using a mirVana miRNA Isolation Kit. RNA quality was assessed on an Agilent 2100 Bioanalyzer. Real-time TaqMan miRNA PCR (Applied Biosystems) was carried out to measure the expression of mature miR-107, miR-21 or miR-15b in untreated cells and cells transfected with either anti-miR-107, anti-miR-21 or anti-miR-15b as control anti-miR. Normalization was performed using the 2-ΔΔCT method relative to 18S RNA. All PCR reactions were performed in triplicate.

**Western blotting**

Cells were seeded into 100 mm plates on the day of transfection at a density of 2–4 × 105 cells. After 72 h, total cell lysate was prepared by washing the cells with phosphate-buffered saline and lysed using buffer containing 50 mM Tris–HCl (pH 7.2), 250 mM sucrose, 2 mM ethylenediaminetetraacetic acid (pH 7.6), 1 mM ethyleneglycol-bis(amoxyethyether)-tetraacetic acid (pH 7.5), 50 mM NaF, 1% Triton X-100, 100 mM sodium orthovanadate, protease inhibitor cocktail and 10 mM β-mercaptoethanol. Protein concentration was determined by the Bradford method. Samples (20–80 μg) were loaded onto 4–20% Tris-Glycine gels (Invitrogen, Carlsbad, CA). After blotting, the membrane was incubated overnight with goat BACE1 antibody at 1:1000 (R&D Systems, Minneapolis, MN), PTEN antibody at 1:1000 (Cell Signaling Technology, Boston, MA) or B-cell lymphoma 2 (Bcl-2) at 1:1000 dilution (Stressgen, Ann Arbor, MI) and horseradish peroxidase linked (Jackson Immunoresearch Laboratories, West Grove, PA) secondary antibody at 1:10,000 dilution and chemiluminescent detection was performed. Unless noted, all other reagents were from Sigma.

**Statistics**

miRNA raw expression values were initially normalized to 18S RNA expression. Two hundred and fifteen miRNAs were disqualified due to near zero levels. The resultant readings were further normalized by the quantile normalization approach (32). Treatment versus control differences in log scale were shown.

Table II. Differentially regulated miRNAs in tumors compared with normal colon mucosa

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Expression ratio tumor/ control</th>
<th>P-value</th>
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<td>miR-224</td>
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<td>miR-223</td>
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<td>miR-146b</td>
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<td>1.00E-06</td>
</tr>
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<td>miR-335</td>
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<td>miR-146a</td>
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<td>miR-142-3p</td>
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<td>1.00E-06</td>
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<td>miR-199a</td>
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<td>miR-142-5p</td>
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<td>1.00E-06</td>
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<tr>
<td>miR-365</td>
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<td>1.60E-03</td>
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<td>miR-199b</td>
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Results

miRNA expression patterns in rat colon adenocarcinoma

Using a Taqman PCR approach, we detected 153 mature miRNAs in normal rat colonic mucosa (Table I). This is the first time a collection of rat intestinal miRNAs has been identified. In order to elucidate the dysregulation of the miRNA network in a highly relevant model of colon carcinogenesis, we compared expression profiles in rats injected with AOM versus saline (control). Specifically, miRNA profiles from six colon tumors were examined relative to normal colonic mucosa using Taqman low-density arrays. As shown in Table II, tumor miRNA profiles were distinctly different in rat tumors. Upon comparison, of the 153 miRNAs expressed in rat colon, 46 were differentially expressed. Twenty-seven were expressed at higher \( P < 0.05 \) levels in tumors with miR-132 enriched to the highest degree at 51-fold. In contrast, 19 miRNAs were expressed at lower \( P < 0.05 \) levels in tumors with miR-215 suppressed the most at 0.17-fold. These data indicate a global perturbation in miRNA expression patterns in AOM-induced colonic tumors.

Diet influences hierarchical miRNA clustering

When the expression of 50 miRNAs was compared between tumors (34 week after carcinogen injection) and AOM-exposed mucosa (10 week), distinct expression patterns were observed (Figure 1). Hierarchical cluster analysis revealed five clear groups: (cluster 1) colonic tumors; (cluster 2) corn oil-cellulose diet + AOM; (cluster 3) fish oil-cellulose diet + AOM; (cluster 4) fish oil-pectin diet + AOM and (cluster 5) corn oil-pectin diet + AOM. As shown in Figure 1, of the AOM-treated groups, the expression pattern in the corn oil/cellulose group was most similar to that of tumors. This observation is similar to our previous findings, in which rats fed corn oil/cellulose and

Fig. 1. Heatmap of standardized colonic miRNA expression profiles. Fifty miRNAs were selected for prediction-mean-square-error reduction using Random Forests software in R. For miRNAs in each AOM group \( (n = 6) \) at 10 weeks, measurements were standardized by subtraction of the expression level using the saline mean values in the same diet group \( (n = 6) \). Tumor data (34 weeks post AOM injection) were subsequently standardized equivalently and clustered into rows and columns. The row variables correspond to miRNA expression values and the column variables are the different diet-treatment groups.
injected with AOM had the highest number of colon tumors of the four diet groups (26). Further examination of the differences in AOM versus saline (control) miRNA expression across diet groups revealed that five miRNAs (let-7d, miR-15b, miR-107, miR-191 and miR-324-5p) were selectively modulated by fish oil exposure (Figure 2). Specifically, for these five miRNAs, expression in the fish oil fed animals was not affected by AOM treatment (AOM:saline ratio ~1.0 or greater), whereas for the corn oil groups, AOM exposure resulted in a significant ($P < 0.05$) downregulation of expression (AOM:saline ratio ~0.8 or less). Expression in tumors for these miRNAs was also significantly ($P < 0.05$) decreased compared with normal colonic mucosa derived from saline-injected (control) animals. These findings are noteworthy in view of the well-documented chemoprotective effects of $n$-3 PUFA (15,16,18–24).

miRNA expression patterns are modulated by fish oil feeding

Colonic mucosa miRNA expression profiles were further examined in AOM-injected rats fed different combinations of fat and fiber relative to saline-treated (control) animals fed the same diets. Overall, fish oil fed animals exhibited the smallest number of differentially expressed miRNAs (Figure 3A). For the corn oil/cellulose diet, 41 miRNAs were differentially expressed (expression in AOM treated divided by saline treated) in carcinogen versus saline-treated groups ($P < 0.05$) at 10 weeks. To explore the pathophysiological relevance of the miRNA profiles, the incidence of adenocarcinomas in 34 week post-carcinogen-injected rats fed the same diet combinations was determined. As expected, the number of tumors was significantly ($P < 0.05$) reduced in fish oil fed animals, reaffirming the chemoprotective properties of $n$-3 PUFA (Figure 3B).

miRNA functional target analysis

Since miRNAs have the potential to regulate tumor suppressors and oncogenes in the colon, the established targets of let-7d, miR-15b, 107, 191 and 324-5p were identified using miRecords (http://mirecords.umn.edu/miRecords), an integrated resource for miRNA–target interactions (31). We specifically avoided inferring miRNA activities, since in silico miRNA target prediction is usually not accurate (35). The established targets for diet-modified miRNAs are shown in Table III. From a cancer perspective, miR-15b has been empirically shown to act as a natural antisense interactor with Bcl-2, a well-documented anti-apoptotic protein (36,37). In addition, CCNE1, encoding cyclin E1, is a direct target in glioma cells (38). This would make miR-15b a putative tumor suppressor. Functional analysis has also demonstrated that miR-107 contributes to the regulation of beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1) and plasminogen activator inhibitor 1 RNA-binding protein (SERBP1) (39,40). SERBP1 has been linked to epithelial cell tumor progression (41).

Colonic miRNA functional target analysis: BACE1 is a target of diet-modulated miR-107

To further assess targets for select diet-modulated miRNAs, we determined whether BACE1 might be a target of miR-107 in colonoocytes. BACE 1 was selected because it is highly expressed in HCT116 human colon cancer cells. Hence, we transfected cells with anti-miR-107 or control anti-miR. On average, transfection efficiency was >70% (supplementary Figure 1 is available at Carcinogenesis Online). Twenty-four hours after knockdown, miR-107 levels were reduced by ~70% (Figure 4). Correspondingly, 72 h after

![Fig. 2. Effect of diet on carcinogen-induced alterations in miRNA expression profiles. Data are shown as a ratio of colonic miRNA expression in AOM compared with the saline injection in both corn oil and fish oil fed rats at 10 weeks ($n = 6$ per treatment). For the five miRNAs shown, expression in the fish oil group was not downregulated by AOM treatment (AOM:saline ratio ~1.0 or greater), whereas for the corn oil group, AOM exposure resulted in a significant ($P < 0.05$) downregulation of expression (AOM:saline ratio ~0.8 or less). Tumor values were derived from adenocarcinomas harvested at 34 weeks relative to saline-injected control.](https://academic.oup.com/carcin/article-abstract/30/12/2077/2391954)

![Fig. 3. Effect of diet on miRNA expression. (A) Venn diagrams showing differential expression of miRNAs in colonic mucosa of rats ($n = 6$) treated with AOM versus saline (control). (B) Bar graph: miRNA expression profiles in colonic mucosa of AOM-injected rats (at 10 weeks) fed different combinations of fat and fiber were compared with animals treated with saline (control) fed the same diets. For example, for the corn oil/cellulose diet, 41 miRNAs were differentially expressed between the AOM and saline-treated groups. For comparison, the number of differentially expressed miRNAs between adenocarcinomas and saline-treated colonic mucosa is also shown (expression in tumor divided by saline for each miRNA). Line graph: Incidence of adenocarcinomas at 34 weeks post-carcinogen injection in rats fed the same four combinations of fat and fiber. Treatment group data points not sharing the same letter are significantly ($P < 0.05$) different.](https://academic.oup.com/carcin/article-abstract/30/12/2077/2391954)
miRNAs and 902 target genes in nine species. Experimentally validated miRNA targets were identified using the miRecords database (http://mirecords.umn.edu/miRecords). This database lists over 301 miRNAs and their established mRNA targets during colon cancer development has not been determined.

PTEN is a target of miR-21 in the colon

miR-21 is a well-known oncogenic miRNA in the colon and was significantly upregulated in rat colon adenocarcinomas compared with normal colonic mucosa (Table II). Since tumor suppressor phospholipid phosphatase (PTEN) has been shown to be a target of miR-21 in gastric cancer cells (37,42), we examined the effect of miR-21 knockdown on its expression (Figure 4). PTEN levels were increased by ~80% in knockdown samples compared with control (Figure 5), confirming that PTEN is a functional target of miR-21 in the colon.

Discussion

It is now appreciated that non-coding RNAs (miRNAs) control translation and mRNA degradation of approximately one-third of the mammalian mRNAs (1,2). Typically, miRNAs (19–27 nt) base pair to the transcripts of protein-coding genes, i.e. gene targets, resulting in downregulation or gene repression. Interestingly, contrary to their traditional role as repressors of gene activation, recent evidence suggests that in certain cases, select miRNAs can switch from repression to activation of protein translation during the cell cycle (44). These findings emphasize the complexity of miRNA-mediated regulation of gene expression. Currently, >500 miRNA genes have been identified, which code for ~700 miRNAs (31). Therefore, the overall goals of this study were to: (i) examine the effects of colon carcinogen on mucosal miRNA expression profiles and (ii) unravel the effects of bioactive dietary components (n-3 PUFA and fiber) on miRNA expression in the colon.

Fig. 4. HCT116 cells were transfected with either (A) 20 nM anti-miR-107, (B) 50 nM anti-miR-21 or (C) 50 nM anti-miR-15b or a control anti-miR. After 24 h, total RNA was isolated and analyzed for miRNA expression as described in Materials and Methods. Asterisk indicates P < 0.05, n = 4 cultures from two separate experiments.
perturbations observed represent changes in miRNAs driving or modulating carcinogenesis.

Although many of the mRNA targets of AOM-modulated miRs are not known, there is extensive evidence that miR-34a contributes to p53-mediated apoptosis and may function as a potential tumor suppressor (57). However, its role in the colon is less clear, with approximately two-thirds of human colon cancer specimens expressing a significant upregulation (58). In comparison, relatively little is known about miR-132 (circadian clock), 223 (myelopoiesis) and 224 (targeting of apoptosis inhibitor-5) (59–61). Our study also provides experimental evidence that the expression levels of miR-215, 194, 375 and 192 are dramatically reduced (0.32-fold or lower) in adenocarcinomas. Interestingly, miR-192 and 215 may function as tumor suppressors, capable of decreasing dihydrofolate reductase expression and suppressing carcinogenesis through p21 accumulation and cell cycle arrest (62–64). In addition, both miR-194 and 375 are regulated by hepatocyte nuclear factor-1α and may play an important role in intestinal epithelial cell differentiation (65,66). Further studies are required in order to validate mRNA targets for these miRs in the colon.

Our experimental protocol also enabled us to quantify subtle dietary effects on miRNA levels in a highly relevant colon cancer model. Consistent with our hypothesis, the n-3 PUFA-enriched chemoprotective diets modulated miRNA signatures in the colon by suppressing the effects of AOM treatment. Although little is known regarding the function of let-7d, miR 15b, 107, 191 or 324-5p, these novel data provide evidence for dietary regulation of miRNA expression in cancer pathogenesis. We also demonstrate for the first time that BACE1 is a target of diet-responsive miR-107 in the colon. Overall, these results are in accordance with recent studies suggesting that
Diverse dietary bioactive components, e.g. butyrate, folate, retinoids and curcumin, exert their effects, in part, through modulation of miRNA expression (67,68). Thus, it is important to elucidate the mechanisms by which n-3 PUFA and other chemoprotective dietary agents alter miRNA levels in the colon.

In summary, we have shown that common global miRNA expression patterns exist in human and rat AOM-induced colon tumors, demonstrating the utility of this model. In addition, chemoprotective n-3 PUFA modulated carcinogen-directed non-coding miRNA signatures. These findings indicate the need to consider the impact of dietary bioactive agents when examining the role of miRNAs in the biology and pathobiology of the gastrointestinal tract.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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References


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