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Western-style diet-induced colonic tumors and their modulation by calcium and vitamin D in C57Bl/6 mice: a preclinical model for human sporadic colon cancer

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We reported previously that a new Western-style diet (NWD) for 18 months, consisting of elevated lipids and decreased calcium, vitamin D and methyl-donor nutrients, induced colonic tumors in normal C57Bl/6 mice [Newmark,H.L. *et al.* (2001) A Western-style diet induces benign and malignant neoplasms in the colon of normal C57Bl/6 mice. *Carcinogenesis*, 22, 1871–1875], suggesting a new mouse model for human sporadic colon cancer. Here, we have extended this study during a longer feeding period of 2 years wherein tumor formation, tumor inhibition by addition of dietary calcium and vitamin D and their effects on gene expression were determined. We also similarly tested individual supplements of methyl donor (transfer) nutrients (folic acid, choline, methionine and dietary fiber), but these had no significant effect on colonic tumor incidence or multiplicity, whereas supplementation with combined calcium and vitamin D produced significant decrease in both colon tumor incidence and multiplicity, during 2 years of feeding. No visible colonic tumors were found at 6 months, very few at 12 months, more at 18 months and significantly at 24 months. In a related study of gene changes of the mouse colonic mucosa at 6 months of feeding taken from this study, long before any tumors were visibly detectable, indicated altered profiles of gene expression linked to later risk of dietary initiation of colon tumor formation. This type of early genetic altered profile, an indication of increased risk of later colonic tumor development, may become a useful tool for prediction of colon tumor risk while the colon grossly still appears histologically and physiologically normal.

Introduction

In a pilot study, we reported previously that a new Western-style diet (NWD) induced benign and malignant neoplasms in normal C57Bl/6 mice (1). The NWD was designed to mimic the suggested colon cancer risk factors in humans: (i) high dietary fat (40% of total calories or 20% of weight of diet); (ii) inadequate dietary calcium (0.5 mg/g diet, ~220 mg/day equivalent in a human 2000 kcal diet) and (iii) inadequate dietary vitamin D_3 (0.11 IU/g diet, approximately equivalent to 50 IU/day in a human 2000 kcal diet). The levels of folic acid, DL-methionine, L-cysteine and choline bitartrate were similarly reduced to the inadequate levels of some human diets, approximately to the lower one-fourth of the average human diets in the USA details of these evaluations and basis for the diet concentrations used have been reported previously (1). The remainder of the diet was based on the normal composition of the AIN-76A diet, designed as a semi-purified diet (2,3).

The results of the first pilot study (1) also indicated that the NWD, essentially using human nutrient-density equivalents of increased dietary fat, coupled with reduced calcium, vitamin D, folate, choline, methionine and fiber induced adenomas and carcinomas in the colon of C57Bl/6 mice without carcinogen exposure or targeted mutations. Generally, these nutritional risk factors correlate with recognized human dietary risk factors for colon cancer.

The study we report now was intended to verify and expand the results of the earlier pilot study in larger groups of animals. In addition, a representative segment of each group of mice was examined at 6 months of feeding, well before any visible pathological or abnormal lesions were seen in the pilot study. These early, apparently normal colons were examined for early genetic alterations by gene expression assay analysis by Dr L.A. (Albert Einstein Cancer Center, Montifiore Medical Center, Bronx, NY) and are reported elsewhere (4).

Abbreviations: GI, gastrointestinal; NWD, new Western-style diet; NWD/CaD, new Western-style diet with increased calcium and vitamin D; NWD/Chln, new Western-style diet with increased choline; NWD/FA, new Western-style diet with increased folic acid; NWD/Fb, new Western-style diet with increased fiber; NWD/Methn, new Western-style diet with increased methionine.

Materials and methods

Mice and diets

A total of 420 C57Bl/6 mice at 3–4 weeks of age of both sexes were obtained from the Jackson Laboratories (Bar Harbor, ME). They were housed 10 mice per cage, with males and females separately, in cages with a wire bottom to prevent coprophagia. Upon arrival, all mice were fed a standard AIN-76A control diet, given water *ad libitum* and kept on a 12 h light–dark cycle in a controlled temperature and humidity room. At 6 weeks of age, these mice were randomly divided into seven diet groups and fed with one of seven diets: (i) AIN-76A diet (control); (ii) NWD; (iii) new Western-style diet with increased calcium and vitamin D (NWD/CaD); (iv) new Western-style diet with increased folic acid (NWD/FA); (v) new Western-style diet with increased choline (NWD/Chln); (vi) new Western-style diet with increased methionine (NWD/Methn) and (vii) new Western-style diet with increased fiber (NWD/Fb) that were continued until the experiment was completed. The body weights were measured biweekly and gastrointestinal (GI) tumor development was monitored by fecal hemoccult test after 12 months of feeding. There were 60 mice per diet group and the mice of each diet group were scheduled to be killed at 12, 18 and 24 months after feeding the diets. After 6 months of feeding, a total of 40 mice with five to eight mice per diet group were killed for microarray assays of the colonic epithelium. Some mice were found dead during 2 years of study. When the experiment was completed, 356 mice remained that were used for analyzing tumor development as indicated in Table I.

Nutritional components of the diets

The nutritional components of the seven diets studied are shown in Table II. The AIN-76A is a semi-purified mouse diet of the American Institute of Nutrition (2,3) used as a control. The other diets were based on AIN-76A diet with indicated modification of nutritional components. The NWD had an increase in lipid content and decreased calcium and vitamin D, fiber and methyl-donor nutrients (folic acid, choline and methionine) to nutrient-density levels approximating those consumed by large segments of human Western populations. The details of the nutritional features of NWD had been described in our previous publication (1). The NWD contains high dietary fat (20% by weight, ~40% of total calories) similar to the USA human diet. NWD represents the low nutrient-density equivalent of USA human intakes of calcium and vitamin D imposed in the standard AIN-76A diet. Fiber was decreased from 5% in AIN-76A, equivalent to 25 g in a human 2000 kcal (500 g dry weight) diet, to 2%, equivalent to ~9 g in a human 2000 kcal diet. Three methyl transfer-donors, folic acid, choline and methionine, and also fiber were decreased in NWD. The other NWD had the same nutrient composition as NWD except for increased levels of calcium and vitamin D in NWD/CaD diet to the nutrient-density equivalents of the maximum levels permitted in USA daily intake. For the remaining four NWDs, each was supplemented with one of four nutritional components that were decreased in NWD, reaching a level slightly higher than in AIN-76A control diet, i.e. folic acid (2.3 versus 2.0 μg/g) in NWD/FA, methionine (0.35 versus 0.3%) in NWD/Methn, choline (0.24 versus 0.2%) and fiber (cellulose, 6 versus 5%) in NWD/Fb

Table I. Number of mice studied

	Overall			12 months			18 months			24 months		
	Total	M	F	Total	M	F	Total	M	F	Total	M	F
AIN-76A	45	27	18	12	9	3	18	12	6	15	6	9
NWD	50	27	23	13	8	5	22	11	11	15	8	7
NWD/CaD	53	29	24	13	7	6	22	13	9	18	9	9
NWD/FA	55	30	25	14	10	4	23	11	12	18	9	9
NWD/Chln	51	28	23	10	8	2	23	11	12	18	9	9
NWD/Methn	52	29	23	18	8	10	18	12	6	16	9	7
NWD/Fb	50	28	22	16	8	8	17	12	5	7	8	9
Total	356	198	158	96	58	38	143	82	61	117	58	59

M, male; F, female.

Table II. Diet compositions

Ingredients % (wt) or amount (wt)	AIN-76A	NWD	NWD/CaD	NWD/FA	NWD/Chln	NWD/Methn	NWD/Fb
Fat (corn oil), %	5 (13)	20 (40)	20 (40)	20 (40)	20 (40)	20 (40)	20 (40)
Calcium, mg/g	5 (2700)	0.5 (220)	7.0 (3000)	0.5 (220)	0.5 (220)	0.5 (220)	0.5 (220)
Vitamin D_3, IU/g	1 (600)	0.11 (50)	2.3 (1000)	0.11 (50)	0.11 (50)	0.11 (50)	0.11 (50)
Phosphorus (PO_4), mg/g	4 (2200)	3.6 (1600)	3.6 (1600)	3.6 (1600)	3.6 (1600)	3.6 (1600)	3.6 (1600)
Fiber (cellulose), %	5 (25)	2 (9)	2 (9)	2 (9)	2 (9)	2 (9)	6 (30)
Folic acid, μg/g	2 (1100)	0.23 (100)	0.23 (100)	2.3 (1000)	0.23 (100)	0.23 (100)	0.23 (100)
DL-Methionine, %	0.3	—	—	—	—	0.36	—
L-Cysteine, %	—	0.3	0.3	0.3	0.3	0.3	0.3
Choline butartrate, %	0.2	0.12	0.12	0.12	0.24	0.12	0.12
kcal/g (approximate)	3.6	4.5	4.5	4.5	4.5	4.5	4.5

Numbers in the table are of units, weight or percent of diet by weight for that specific ingredient per gram of diet. Numbers in parenthesis in the table are the calculated equivalents, based on nutrient density, in a daily human 2000 kcal diet (500 g per dry weight).

(Table II). These increases over the levels in the AIN-76A control diet were designed to balance the increased total nutrient density of the NWD compared with AIN-76A diet as indicated in Table II.

Study of tumor development

To evaluate the effects of different nutritional components on tumor development in the GI tract of normal C57Bl/6 mice, three tumor end points including incidence, multiplicity and histological type of intestinal tumor were used. The whole GI tract and internal organs were removed from each mouse after killing at each time point. The stomach and intestine were fixed in 10% buffered formalin after the lumen was opened. The gross specimens were examined under a dissecting microscope for tumors. The number and location of tumors in the GI tract were recorded. All tumors found were sampled for tissue processing and embedded in paraffin. Histological study was carried out on hematoxylin and eosin-stained slides. Histological typing of intestinal tumors was mainly based on the criteria of World Health Organization. The tumors of extra-GI tract were also studied if any was found.

Immunohistochemistry study for protein expression in tumors

Apc and β-catenin protein expression was carried out in intestinal tumors by immunohistochemistry. Antibodies to *Apc* (C-20, 1/100) and β-catenin (C-18, 1/100) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and applied to tumor tissue sections with avidin–biotin peroxidase technique (ABC kit from Vector Laboratories, Burlingame, CA). Color development was in 3,3′-diaminobenzidine (Sigma, St Louis, MO). Location of the protein expression in tumor cells was particularly defined.

Statistical study

Kruskal–Wallis tests were used for multiple comparisons among seven diet groups. Fisher exact probability (two tailed) and Mann–Whitney test and binomial calculation were used for comparisons between two diet groups. Significant difference was considered when $P < 0.05$.

Results

Body weight

The body weights of mice in each group increased gradually during feeding. The mean body weight in AIN-76A control group was ∼25 g after 4 months of feeding. In general, the mice fed NWDs gained more body weight than mice fed AIN-76A after 5 months of feeding, reaching ∼50 g at 12 months, then remained at the higher level until the experiment was completed, presumably due to a higher NWD nutrient density from the high lipid content as observed previously (1). No significant differences of body weights among the six NWD groups of mice were noted during the experiment.

During 24 months of feeding, tumors were found in the GI tract and from GI tract of C57Bl/6 mice fed different diets. They are described separately below.

Tumorigenesis in the GI tract

A total of 99 intestinal tumors were found from the mice studied over 24 months with total tumor numbers of 13, 32 and 54 at the three time points. They were all studied for histological type. Among 99 tumors, 11% were adenocarcinomas and 89% were adenomas. Adenocarcinomas were only 3% (2 of 58) in the small intestine and 22% (9 of 41) in the right side of colon. Among adenocarcinomas, only three were fully invasive carcinomas including two in NWD/Methn and one NWD/Fb group. The other nine were early invasive carcinomas involving the submucosa. Seventy-three percentage of the adenocarcinomas occurred after 18 months of feeding. Tubular adenomas were often seen, especially in the small intestine. Flat adenomas were also observed in the cecum. In this report, we cannot compare the effects of dietary nutritional components on tumor progression due to the limited number of each histological type of tumors in each diet group and at each time point. Actually, both adenomas and carcinomas were seen in each diet group. This restriction allowed us to focus primarily on the tumor incidence and multiplicity in the small intestine and colon and to analyze the effects of dietary nutritional components on tumor development. Only one microadenoma was found in the stomach of a mouse fed NWD for 18 months; for convenience in analysis, this was included in the total of small intestine. Therefore, the data reported here were mainly tumor incidence and multiplicity in the small intestine and colon of seven diet groups.

After 6 months of feeding, a few mice from each group were killed and examined for visible lesions in the intestinal tract. None were found. These early, apparently normal colons were examined for early genetic alterations, by gene expression analysis, of the flat colonic mucosa induced by diet at 6 months (4). The results indicated that feeding the NWD generated significantly altered profiles in gene expression in the flat mucosa, which also inhibited heterogenicity among the mice similar to that initiated in the mucosa by inheritance of mutant Apc allele (4).

After 12 months of feeding, there were no intestinal tumors in mice fed AIN-76A and NWD. The remaining groups had tumors in the small intestine and/or in colon. The overall tumor incidence was 15% in NWD/CaD, 7% in NWD/FA, 20% in NWD/Methn, 22% in NWD/Chln and 6% in NWD/Fb. There were no tumors in the colon of three diet groups, NWD/CaD, NWD/FA and NWD/Fb. For the other two groups, NWD/Methn and NWD/Chln, the tumor incidence was similar in both small intestine and colon with 10% in NWD/Methn and 17% in NWD/Chln. There was no statistical significant difference of tumor incidence either in small intestine or in colon between groups of AIN-76A and NWD or between NWD and NWD with any of the added supplements at 12 months of feeding. The mean tumor multiplicity showed no significant difference in all five groups found with intestinal tumors, compared with NWD, except for NWD/Methn group. At 12 months, the tumor multiplicity increase was significant in the overall intestine (0.39, $P < 0.01$), the colon (0.22, $P < 0.05$) and not fully significant in the small intestine (0.17, $P = 0.08$) compared with NWD (0.00) (Tables III and IV).

After 18 months of feeding, intestinal tumors developed in both small intestine and colon of mice in every diet group of the seven diet groups studied including AIN-76A, NWD and NWD with supplements. In the colon, the tumor incidence and tumor multiplicity was low in the seven diet groups. But it showed a trend suggesting that the tumor multiplicity increased in NWD compared with AIN-76A (0.23 versus 0.06, $P > 0.05$) and decreased in NWD/CaD (0.05 versus 0.23, $P > 0.05$). There was no significant difference of tumor incidence and multiplicity overall in the total intestine, or in the small intestine and colon individually between groups of NWD and AIN-76A, and between groups of NWD and NWD with supplements (Tables III and IV).

At 18 months of feeding, this study failed to show significant increase in the intestinal tumors in the NWD as demonstrated in the pilot study (1), but did show this effect at 24 months. Other than normal biological and perhaps seasonal and experimental variability, we have no real explanation for the difference in the 18 months time period.

After 24 months of feeding, a statistically borderline difference was seen in tumor multiplicity overall in the intestine ($P = 0.08$) and in the colon ($P = 0.08$) among the seven diet groups. NWD increased intestine tumor incidence overall (53 versus 27%, $P > 0.05$) and in the colon by 2.9-fold (27 versus 7%, $P > 0.05$) compared with AIN-76A controls. The total tumor multiplicity increase was also observed in the intestine by 1.5-fold (0.67 versus 0.27, $P = 0.08$) and in the colon by 3.7-fold (0.33 versus 0.07, $P > 0.05$). However, the NWD with increased calcium and vitamin D significantly inhibited tumor development in the intestine. The tumor incidence decreased by 89% (6 versus 53%, $P < 0.01$) overall in the intestine and no tumors in the colon (0 versus 27%, $P < 0.05$) of mice in NWD/CaD compared with NWD group. Calcium and vitamin D supplement also decreased the tumor multiplicity by 91% overall in the intestine (0.06 versus 0.67, $P < 0.01$) and by 82% in the small intestine (0.06 versus 0.33, $P < 0.02$) and in the colon (0.00 versus 0.33, $P < 0.02$) compared with NWD. Tumor development in the intestine was not inhibited by any of the other supplements modified in NWD, including folic acid, methionine, choline and fiber (Tables III and IV).

These results indicate that a NWD with increased lipid and decreased nutrients of calcium, vitamin D, methyl donors and fiber produced intestinal tumor development in wild-type C57Bl/6 mice after long-term feeding. The colonic tumors caused by the NWD were significantly inhibited by increased dietary calcium and vitamin D content, but not by any individually increased nutrient of folic acid, methyl donors (methionine and choline) or fiber. In this study, increased folic acid, methionine, choline or dietary fiber did not inhibit the increased development of colon tumors induced by the NWD, a mimic of the human NWD. However, increased dietary calcium and vitamin D completely inhibited such colon tumor development.

Table III. Tumor incidence in small and large intestine of C57Bl/6 mice at three time points during 24 months of feeding

	Number of mice studied	Number (%) mice with tumors		
		Overall	Small intestine	Colon
12 months				
AIN-76A	12	0 (0)	0 (0)	0 (0)
NWD	13	0 (0)	0 (0)	0 (0)
NWD/CaD	13	2 (15)	2 (15)	0 (0)
NWD/FA	14	1 (7)	1 (7)	0 (0)
NWD/Chln	10	2 (20)	1 (10)	1 (10)
NWD/Methn	18	4 (22)	3 (17)	3 (17)
NWD/Fb	16	1 (6)	1 (6)	0 (0)
18 months				
AIN-76A	18	4 (22)	4 (22)	1 (6)
NWD	22	3 (14)	2 (9)	1 (5)
NWD/CaD	22	3 (14)	2 (9)	1 (5)
NWD/FA	23	7 (30)	6 (26)	1 (4)
NWD/Chln	23	3 (13)	3 (13)	2 (9)
NWD/Methn	18	4 (22)	2 (11)	2 (11)
NWD/Fb	17	1 (6)	0 (0)	1 (6)
24 months				
AIN-76A	15	4 (27)	4 (27)	1 (7
NWD	15	8 (53)	4 (27)	4 (27)
NWD/CaD	18	1 (6)[a]	1 (6)	0 (0)[b]
NWD/FA	18	8 (44)	4 (22)	5 (28)
NWD/Chln	18	6 (33)	6 (33)	1 (6)
NWD/Methn	16	6 (38)	4 (25)	3 (19)
NWD/Fb	17	6 (35)	2 (12)	5 (29)

By Fisher exact probabilities test (two tailed) compared with AIN-76A: no significant difference with NWD; compared with NWD: [a]$P < 0.01$, [b]$P < 0.04$.

Tumorigenesis outside the GI tract

Besides tumors in the intestine, extra-GI tract tumors were observed in C57Bl/6 mice after long-term feeding of the diets studied described above. In seven diet groups, a total of 48 (14%) mice had extra-GI tumors including three (1%) in AIN-76A group and 45 (13%) in NWD groups among 356 mice over the 24 months studied. Most of the extra-GI tumors were observed after 18 months of feeding, except for one mouse in AIN-76A group. Four major types of extra-GI tumors were found: 4.2% with lung tumors (alveolar/bronchiolar adenomas/carcinomas), 3.9% with non-Hodgkin's lymphomas, 2.8% soft tissue tumors (hemangioma and leiomyosarcoma) and 2.8% with liver tumors (hepatoblastoma and Kupffer cell sarcoma). The incidence of lung tumors, non-Hodgkin's lymphoma and soft tumors, but not liver tumors, was higher in NWD than AIN-76A (all $P > 0.05$). It was interesting to note that the incidence of extra-GI tumors was also higher in NWD with supplementation of methyl donors than in NWD alone. This is similar to the effect of long-term effect of increased folic acid over 7 years observed in a human study by Cole *et al.* (5). There were no soft tissue tumors in the NWD/Fb group and no liver tumors in the NWD/Methn group. The extra-GI tumors showed no statistical significant difference between NWD and AIN-76A.

The data of 24 months as indicated in Table III illustrates the potential relation between GI tumors and diet in this study group. A suggested effect in the test is that increased calcium and vitamin D reduced the increased tumors of NWD addition by itself (paragraph starting with 'after 24 months of feeding…').

Table IV. Tumor multiplicity in the small and large intestine of C57/Bl6 mice at three time points during 24 months of feeding

	N	Number of tumors per mouse		
		Overall	Small intestine	Large intestine (colon)
12 months				
AIN-76A	12	0.00 ± 0.00 (0–0)	0.00 ± 0.00 (0–0)	0.00 ± 0.00 (0–0)[A]
NWD	13	0.00 ± 0.00 (0–0)	0.00 ± 0.00 (0–0)	0.00 ± 0.00 (0–0)
NWD/CaD	13	0.15 ± 0.10 (0–1)	0.15 ± 0.10 (0–1)	0.00 ± 0.00 (0–0)
NWD/FA	14	0.07 ± 0.07 (0–1)	0.07 ± 0.07 (0–1)	0.00 ± 0.00 (0–0)
NWD/Chln	10	0.20 ± 0.13 (0–1)	0.10 ± 0.10 (0–1)	0.10 ± 0.10 (0–1)
NWD/Methn	18	0.39 ± 0.20 (0–3)[a]	0.17 ± 0.09 (0–1)[b]	0.22 ± 0.13 (0–2)[c]
NWD/Fb	16	0.06 ± 0.06 (0–1)	0.06 ± 0.06 (0–1)	0.00 ± 0.00 (0–0)
18 months				
AIN-76A	18	0.28 ± 0.14 (0–2)	0.22 ± 0.10 (0–1)	0.06 ± 0.06 (0–1)
NWD	22	0.32 ± 0.23 (0–5)	0.09 ± 0.06 (0–1)	0.23 ± 0.23 (0–5)
NWD/CaD	22	0.14 ± 0.08 (0–1)	0.09 ± 0.06 (0–1)	0.05 ± 0.05 (0–1)
NWD/FA	23	0.30 ± 0.10 (0–1)	0.26 ± 0.09 (0–1)	0.04 ± 0.04 (0–1)
NWD/Chln	23	0.22 ± 0.13 (0–2)	0.13 ± 0.07 (0–1)	0.09 ± 0.06 (0–1)
NWD/Methn	18	0.22 ± 0.10 (0–1)	0.11 ± 0.08 (0–1)	0.11 ± 0.11 (0–1)
NWD/Fb	17	0.06 ± 0.06 (0–1)	0.00 ± 0.00 (0–0)	0.06 ± 0.06 (0–1)
24 months				
AIN-76A	15	0.27 ± 0.15 (0–2)[B]	0.20 ± 0.11 (0–1)	0.07 ± 0.15 (0–1)[C]
NWD	15	0.67 ± 0.19 (0–2)[1]	0.33 ± 0.13 (0–1)	0.33 ± 0.16 (0–2)
NWD/CaD	18	0.06 ± 0.06 (0–1)[a]	0.06 ± 0.06 (0–1)[c]	0.00 ± 0.00 (0–0)[c]
NWD/FA	18	0.56 ± 0.17 (0–2)	0.22 ± 0.10 (0–1)	0.22 ± 0.10 (0–2)
NWD/Chln	18	0.44 ± 0.19 (0–3)	0.39 ± 0.14 (0–2)	0.06 ± 0.06 (0–1)[d]
NWD/Methn	16	0.63 ± 0.27 (0–4)	0.44 ± 0.26 (0–4)	0.19 ± 0.10 (0–1)
NWD/Fb	17	0.59 ± 0.30 (0–5)	0.18 ± 0.13 (0–2)	0.41 ± 0.19 (0–3)

N, number of mice studied. Mean ± SEM (range). By Kruskal–Wallis test multiple comparison among seven groups: $^{A}P = 0.096$; $^{B}P = 0.084$; $^{C}P = 0.078$. By Mann–Whitney test or binomial calculation, compared with AIN-76A, $^{1}P = 0.079$; compared with NWD, $^{a}P < 0.01$; $^{b}P = 0.073$; $^{c}P < 0.02$; $^{d}P < 0.098$. The numbers in parentheses indicate the range of tumor numbers per mouse in each group.

Discussion

This larger scale study of NWD, which is a mimic of the high dietary fat and reduced calcium, vitamin D, folic acid, choline and methionine of the USA human NWD, confirms and extends the results of the previous pilot study (1). Long-term feeding of the NWD for 2 years to mice induced colon tumors in ∼25% of the mice (Tables III and IV). The use of long-term feeding of the NWD to C57Bl/6 mice thus represents a new experimental colon cancer model, which closely duplicates natural human colon cancer development, without the use of toxic carcinogens or other artificial stimuli.

The NWD, when corrected for its inadequate calcium and vitamin D content, in the mouse model generally fails to develop more colon tumors than AIN-76A diet (Tables III and IV). This strongly suggests that increasing USA human dietary calcium and vitamin D intake could produce a significant colon cancer reduction in the USA population, especially if this doable increase starts early in life. A recent report of a randomized, placebo-controlled, 4 year, population-based, double-blind study in healthy post-menopausal women in rural Nebraska indicated that improving calcium and vitamin D dietary status reduces all-cancer risk (6) in accordance with this rodent study.

Folic acid, by inadequacy or supplementation did not produce measurable effects on colon cancer development in this dietary mouse model of colon tumorigenesis (Tables III and IV). Supplementary choline or methionine in the NWD did, however, modestly reduce colon tumor development (Tables III and IV). These results in mice are comparable with a report of colon polyp recurrence in humans given a dietary daily supplement of 1000 μg folic acid, which showed no reduction in 4 years, but an increased risk of multiple adenomas in ∼7 years of intervention (5). Our results with NWD supplemented with choline or more effectively by methionine, metabolically related to folic acid in methyl transfer systems, however, did show some reduction in colon tumor development in mice by NWD (Tables III and IV). Tumor development in the small intestine in contrast was not reduced by choline or methionine in this study. While not identical to the results in the colon, the basic results of increased tumor risk of the NWD on 2 years of feeding mice, and reduction of risk by supplementation with adequate calcium and vitamin D, were also present in the small intestine (Tables III and IV). Overall tumor incidence over 24 months induced by NWD was largely prevented by supplementation with adequate dietary calcium and vitamin D. This observation in mice is comparable with the human study by Lappe *et al.* (6), indicating that vitamin D and calcium dietary supplementation reduces total cancer risk in a short (4 year) randomized trial.

This study in mice can be summarized as follows:

(i) The NWD, a mimic of the human NWD, with high dietary fat, reduced dietary calcium and vitamin D, can induce colon tumors on long-term feeding to normal wild-type mice, without the use of any chemical carcinogen. This represents a useful model of mouse colon tumorigenesis for the study of colon cancer prevention for human application. It also represents a confirmation of our previous pilot study of the NWD (1).

(ii) Calcium and vitamin D dietary intake, when adequate, can have a strong effect in reduction of colon tumor development in mice on regular intake of a NWD.

(iii) Folic acid diet supplementation, or diet supplementation with methionine or choline, metabolically related to folic acid, have no preventive effect on colon tumor induction by the NWD.

(iv) We strongly suggest further human studies of supplemental dietary calcium and vitamin D to reduce the risk of colon tumor development.

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References

1. Newmark,H.L. *et al*. (2001) A Western-style diet induces benign and malignant neoplasms in the colon of normal C57Bl/6 mice. *Carcinogenesis*, **22**, 1871–1875.
2. Bierei,J.G., Stoewsand,G.S., Briggs,G.M., Phillips,R.W., Woodard,J.C. and Knapka,J.J. (1977) American Institute of Nutrition. AIN report of the AIN *ad hoc* committee on standards for nutritional studies. *J. Nutr.*, **107**, 1340–1348.
3. Bierei,J.G. (1980) AIN second report of the AIN *ad hoc* committee on standards for nutritional studies. *J. Nutr.*, **110**, 1726.
4. Yang,K. *et al*. (2008) Dietary induction of colonic tumors in a mouse model of sporadic colon cancer. *Cancer Res.*, **68**, 7803–7810.
5. Cole,B.F. *et al*. (2007) Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. *JAMA*, **297**, 2351–2359.
6. Lappe,J.M. *et al*. (2007) Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. *Am. J. Clin. Nutr.*, **85**, 1586–1591.

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