# Colon Cancer Risk of a Westernized Diet Is Reduced in Mice by Feeding Cruciferous or Apiaceous Vegetables at a Lower Dose of Carcinogen but Not a Higher Dose

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Western-style diets (WD) are associated with greater risk of colon cancer. Exposure to 2-amino-1-methyl-6-phenylimidazo(4,5-b) pyridine (PhIP), a food-borne carcinogen, is linked to increased colon cancer risk. In contrast, intake of apiaceous and cruciferous vegetables (APIs and CRUs) is associated with reduced risk. Here we evaluated effects of a WD alone or a WD containing API or CRU, relative to a purified diet (basal), on colon cancer risk in mice. All diets were fed at one of two concentrations of PhIP (100 or 400 ppm). The activity of the hepatic PhIP-activating enzyme, cytochrome P450 (CYP) 1A2, was examined at week 4 and colonic precancerous lesions (aberrant crypt foci, ACF) were enumerated at week 12. In low PhIP-fed groups, CYP1A2 activity was greater for CRU than all other groups, which did not differ from one another. WD had a significantly greater effect on the formation of ACF than the basal diet. In groups fed API or CRU, the ACF number was reduced to the level observed in the basal diet-fed group. In high PhIP-fed groups, all WD-based diets had greater CYP1A2 activity than the basal diet-fed group. Surprisingly, the basal diet group had more ACF than the WD group, and API and CRU groups did not differ from the WD alone group. Thus, at the lower dose of PhIP, the WD increased colon cancer risk in mice, compared to a purified diet, and APIs and CRUs reduced the risk of the WD. However, at the higher dose of PhIP, the enhancement of colon cancer risk by the WD was not evident, nor was the chemopreventive effect of these vegetables.

**Key Words** Colonic neoplasms, Western diet, Heterocyclic aromatic amines, Vegetables, Mice

## INTRODUCTION

Colon cancer is the third leading cause of cancer-related deaths in the United States (US) and other Western societies [1], and the incidence of colon cancer is rapidly increasing in countries that are adopting Western lifestyles and dietary habits [2,3]. Epidemiological studies strongly associate a Western-style diet (WD), characterized by consumption of large amounts of red and processed meat, refined grains, and sugar-containing foods and low amounts of fruits and vegetables, with this increased incidence, recurrence, and mortality of colon cancer incidence [4,5]. However, it remains uncertain which food factors are most influential in altering colon cancer risk, and the mechanism by which these foods may alter this risk is still under investigation.

WDs for animal studies have been developed to examine the effect of individual foods or food components on cancer risk. For example, Newmark et al. [6] formulated a diet, termed the New Western Diet, which resulted in a large percentage of mice developing tumors after 18 months, even without administration of a carcinogen. However, this diet was extremely low in both calcium and vitamin D, providing only approximately 20% of the calcium needed to maintain normal bone growth [7], and less than 10% of the vitamin D to achieve maximal serum concentrations of 25-dihydroxyvitamin D3 [8], levels which caused deficiencies of calcium and vitamin D in other studies [7,9]. Another commonly used rodent diet used to mimic a WD is a high-fat diet, which increases the risk of colon cancer in mice [10] and rats [11]. However, compared with a typical Western dietary pattern,

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the high-fat diets contain far more sugar and fat than the typical American diet [10,12]. Since the New Western Diet does not reflect the typical dietary pattern of Americans and high fat WDs do not approximate the average macro- and micronutrient intakes of Americans, there is a challenge in translating the results from animal studies to humans using these diets. However, a new rodent diet, termed the Total Western Diet (TWD) created based on used National Health and Nutrition Examination Survey dietary intake information, matches the average macro- and micronutrient diet composition of Americans [12]. Compared to the commonly used purified rodent AIN-93G diet, the TWD contains more saturated and monounsaturated fats, simple sugars (2-fold), and sodium and less polyunsaturated fat, complex carbohydrates, calcium, copper, folate, thiamine, and vitamins $B_6$, $B_{12}$, D, and E [12]. Thus, the TWD is of interest as a background diet for studies of the effect of dietary components on colon cancer risk.

Recently, The Working Group of the International Agency for Research on Cancer designated red meat consumption as "probably carcinogenic to humans" (Group 2A), based on epidemiological evidence of a positive association between red meat consumption and colorectal cancer [13]. Although the component(s) of red meat that may be responsible for this elevation in risk is uncertain, one class of compounds implicated are heterocyclic aromatic amines (HAAs). HAAs, which are formed in meat and fish cooked at high temperature, are known carcinogens. 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) is the predominant HAA formed in overcooked meats, and has been shown to be a colon carcinogen in both male and female F344 rats [14]. PhIP also induces colonic precancerous lesions (aberrant crypt foci, ACF) in female C57BL/6 mice [15], including dysplastic ACF, which are the subset of ACF most likely to progress to tu-

mors [16]. Humans consuming a WD are habitually exposed to PhIP [17], and epidemiological evidence suggests that a greater intake of PhIP is associated with an increased risk of colorectal adenoma [18].

Epidemiological studies investigating the relationship between colon cancer risk and intake of vegetables have varied in their conclusions of the strength of the evidence for a protective effect of vegetables. Boeing et al. [19] judged the evidence for protection as probable, Aune et al. [20] described the relationship between vegetable consumption and colon cancer as weak and nonlinear, and the World Cancer Research Fund/American Institute for Cancer Research stated the evidence as only limited-suggestive [21]. However, one class of vegetables, cruciferous vegetables (CRUs), is strongly associated with protection in observational studies [22]. This chemopreventive association has been confirmed experimentally in carcinogen-treated rats fed CRUs (e.g., cabbage, watercress, and broccoli), who had fewer total and dysplastic ACF compared to animals fed a vegetable-free diet [23]. Further, we have shown that when vegetables were fed only after carcinogen administration (post-initiation stage), CRU feeding decreased the number of ACF, the number of β-catenin accumulated ACF (BCACF), a marker of dysplasia, and expression of DCLK1 within ACF, a putative cancer stem cell marker in the context of inflammation [24]. Apiaceous vegetables (APIs; e.g., celery and parsnip) are another class of vegetables that may be chemopreventive. Feeding APIs reduced the proportion of dysplastic ACF and DCLK1 expression within ACF [24]. We have also demonstrated that in rats administered PhIP, those adapted to a diet containing APIs had fewer colonic PhIP-DNA adducts compared to animals fed a vegetable-free diet [25]. Given the evidence that these two classes of vegetables are chemopreventive, it was of
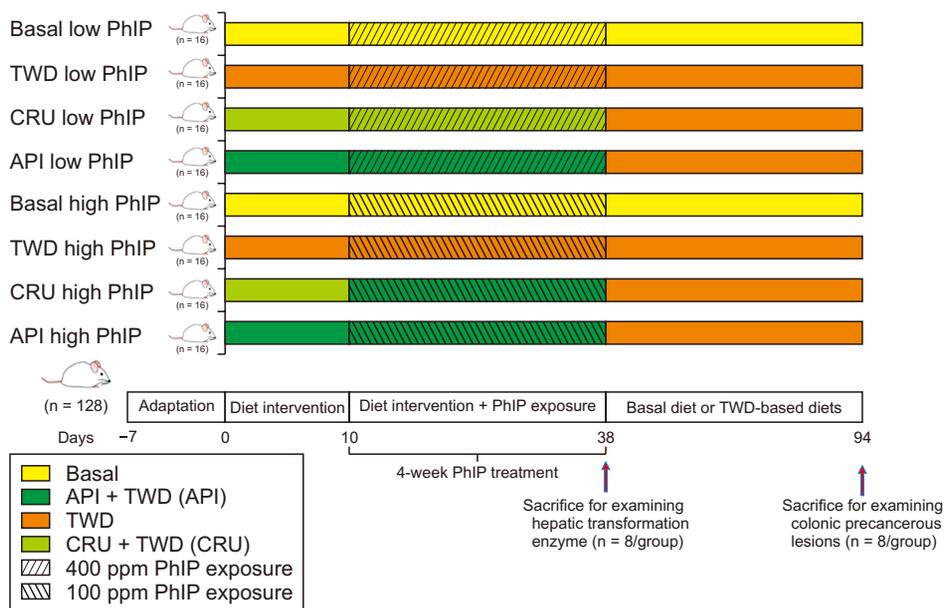


Figure 1. Experimental design. API, apiaceous vegetable; TWD, total Western diet; CRU, cruciferous vegetable; PhIP, 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine.

interest to examine their potential to reduce colon cancer risk in the context of a WD, using a foodborne carcinogen. Thus, the objectives of this study were to assess in mice fed PhIP the effects of APIs and CRUs, fed as part of a TWD, on colon cancer risk markers and on activities of hepatic biotransformation enzymes involved in PhIP activation and detoxification.

## MATERIALS AND METHODS

### Animals and treatments

One hundred twenty-eight male A/J mice (average body weight 18 g) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animals were housed four per cage with corncob bedding and kept on a 12-hour light/dark cycle at 23°C. Animals were given water and diet ad libitum during the study. All animal use procedures were approved by the University of Minnesota Committee on Animal Care and Use and were consistent with Public Health Service Policy on the Humane Care and Use of Laboratory Animals (Protocol ID: 1411-31998A).

After a week adaptation to a purified rodent diet (AIN-93G; basal), the mice were randomized into 8 groups (n = 16 mice per group) and fed the experimental diets without and with PhIP for 10 days and 4 weeks, respectively (Fig. 1). The PhIP was kindly donated by Dr. Robert Turesky of the University of Minnesota. The four experimental diets were as follows: (1) basal, (2) TWD, (3) TWD containing 21% APIs, and (4) TWD containing 21% CRUs. This concentration of dietary vege-

tables was chosen as previous studies had demonstrated a reduction in colonic PhIP-DNA adducts at this same concentration [25]. The composition of the diets is shown in Table 1 and the composition of the fat sources used in the Basal and TWD are shown in Table S1. The mineral and vitamin mix compositions of the Basal and TWD diets are shown in Tables S2 and S3, respectively. All diets used in this study were prepared and maintained as previously described [16,24]. After 4 weeks on the assigned diets containing PhIP, 8 mice from each group were euthanized by $CO_2$ asphyxiation for examination of hepatic biotransformation enzyme activities. The remaining animals were fed their respective diets for an additional 8 weeks but without added PhIP (the basal diet continued to the basal diet; TWD or the vegetable-containing TWDs changed to TWD) (Fig. 1). At the end of week 12, all remaining mice were euthanized by $CO_2$ asphyxiation for examination of ACF.

### Hepatic microsomal preparation, protein quantification, and measurement of biotransformation enzyme activity

Hepatic microsomal preparation, protein quantification, and microsomal cytochrome P450 (CYP) 1A2 and UDP-glucuronosyltransferase 1A (UGT1A) activities were determined as previously described [25].

### Determination of aberrant crypt and ACF

Aberrant crypt (AC) and ACF were determined as previously described [24].

Table 1. Diet ingredients of vegetable-free basal, TWD, and vegetable-containing TWD

| Constituent (g/kg) | Basal | TWD | Cruciferous-vegetable containing TWD | Apiaceous-vegetable containing TWD |
|---|---|---|---|---|
| Cornstarch | 397.5 | 230 | 224.31 | 214.82 |
| Casein | 200 | 190 | 185.5 | 188 |
| Dextrinized cornstarch | 132 | 70 | 70 | 70 |
| Sucrose | 100 | 261.4 | 261.4 | 261.4 |
| Oil or oil mixture[a] | 70 | 165.35 | 164.95 | 164.856 |
| Fiber | 50 | 30 | 26.1 | 23.17 |
| Mineral mix[b] | 35 | 35 | 35 | 35 |
| Vitamin mix[c] | 10 | 10 | 10 | 10 |
| L-cystine | 3 | 2.85 | 2.85 | 2.85 |
| Choline bitartrate | 2.5 | 1.4 | 1.4 | 1.4 |
| Sodium chloride | 0 | 4 | 4 | 4 |
| Cruciferous vegetables[d] | 0 | 0 | 210 | 0 |
| Apiaceous vegetables[d] | 0 | 0 | 0 | 210 |
| Total | 1,000 | 1,000 | 1,195.51 | 1,185.496 |
| % Carbohydrate[e] | 62.95 | 56.14 | 56.42 | 56.72 |
| % Protein[e] | 20.00 | 19.00 | 18.96 | 18.96 |
| % Fat[e] | 7.00 | 16.54 | 16.51 | 16.51 |
| % Dietary fiber[e] | 5.00 | 3.00 | 2.99 | 2.99 |

TWD, total Western diet. [a]Oil ingredients of basal and TWD are described in Table S1. [b]Mineral mix ingredients of basal and TWD are tabulated in Table S2. [c]Vitamin mix components of basal and TWD are shown in Table S3. [d]Fresh weight of the vegetable. [e]Carbohydrate, protein, fat, and dietary fiber from vegetables were calculated after subtracting their water content, as indicated in the United States Department of Agriculture food composition database.

## β-catenin immunohistochemistry, image acquisition, and analysis

Colonic β-catenin immunohistochemistry from animals fed the low PhIP-containing diets was determined as previously described [24]. The expression area of β-catenin within ACF from animals fed the low PhIP-containing diets was evaluated by semi-automated analysis using ImageJ software, Color Deconvolution for H-DAB Images (NIH), as previously described [24].

## Datamining public clinical datasets containing gene expression profiling data of colorectal adenocarcinomas from American and Japanese subjects

The expression of CTNNB1 (the gene for β-catenin) and APC (upstream of β-catenin in WNT signaling) in human colorectal adenocarcinomas was assessed by the Gene Expression Omnibus (GEO), a NCBI public repository of functional genomics data. All datasets involving studies of colorectal adenocarcinoma in Americans and Japanese patients (GSE18105 [26], GSE28000 [27], and GSE44861 [28]) were queried. The GSE28000 and GSE 44861 are the colorectal adenocarcinoma data of White & African American patients (normal = 24, cancer = 24) and White American patients only (normal = 48, cancer = 48), respectively. The GSE18105 (normal = 17, cancer = 17) contains the colorectal adenocarcinoma data of Japanese patients. The CTNNB1 and the APC gene data were downloaded and compared among White & African American patients, White American patients only, and Japanese patients.

## Statistical analysis

Our primary interest was to test how the colon cancer risk (primarily evaluated by ACF number) was affected with respect to diet types and PhIP concentrations. To determine the appropriate sample size, we used G Power software [29] and compared the number of ACF in 8 groups via ANOVA (repeated measures, within-between interactions) with a type I error rate of $\alpha$ = 0.025 (one-sided significance level due to the characteristic of ANOVA) and power of $1-\beta$ > 0.8 (80%). The calculated effect size f was 0.211 for the number of ACF in accordance with the diet types and the concentrations of PhIP. Based on the power analysis, total sample size should be 104 for 8 groups (13 mice per group). The final sample size used was 16 mice per group (128 total mice).

Results were analyzed by two-way ANOVA using PROC MIXED in SAS System for Windows release 9.4 (SAS Institue Inc., Cary, NC, USA), with diet and PhIP dose, and their potential interaction, as fixed effects for hepatic biotransformation enzyme activity and ACF determination, with cage as a random effect. When a statistically significant difference ($P \leq 0.05$) was found, differences among diets were inspected using Duncan's multiple range test, for which the probability value for statistical significance was set at $P < 0.05$. The data for BCACF was semi-continuous, as there were values of zero. Thus, this parameter was evaluated with a zero-inflated gamma model using PROC FMM in SAS (SAS Institute Inc.), employing the maximum likelihood estimation method. The associations between morphological and biochemical markers were analyzed by Pearson *rs.* The differences in the CTNNB1 and APC genes from GEO datasets were analyzed by one-way ANOVA with Duncan's multiple range test ($P < 0.05$). Statistical analyses were performed using SAS System
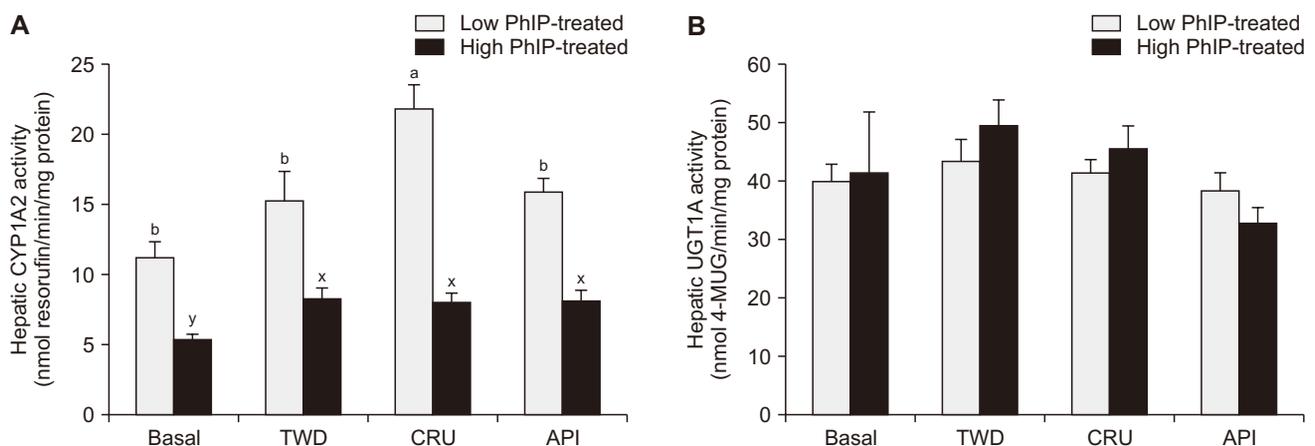


**Figure 2. Effects of TWD and API- and CRU-containing TWD on hepatic enzymatic activities of (A) CYP1A2 and (B) UGT1A in the low PhIP-treated groups (bright grey) and in the high PhIP-treated groups (dark grey).** Values are presented as mean ± SEM; n = 8. Within a parameter, bars that do not share a letter are significantly different ($P < 0.05$) as determined by two-way ANOVA with Duncan's post-hoc test. (A) There were statistically significant main effects of diet ($P < 0.001$) and PhIP ($P < 0.001$) and a statistically significant interaction ($P = 0.009$) for hepatic CYP1A2 activity by two-way ANOVA. (B) There was a trend for a main effect of diet ($P = 0.097$), but no main effect of PhIP, and no interaction for hepatic UGT1A activity by two-way ANOVA. CYP, cytochrome P450; TWD, total Western diet; CRU, cruciferous vegetable; API, apiaceous vegetable; PhIP, 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine; UGT1A, UDP-glucuronosyltransferase 1A.

for Windows release 9.4 (SAS Institute Inc., Cary, NC, USA).

## RESULTS

### Food intake and weight gain

Body weight of the mice did not differ significantly among the diet groups throughout the study. Basal diet-fed mice in the high PhIP-fed group consumed significantly greater amounts of diet than the TWD and API-containing TWD groups. However, as the TWD (18.79 kJ/g diet) and API-containing TWD (18.41 kJ/g diet) had a greater energy density than the basal diet (16.53 kJ/g diet), a comparison based on energy intake among the groups was done. Energy intake did not differ significantly among the groups. Final body weight, food intake, and energy intake are summarized in Table S4.

### Hepatic biotransformation enzyme activity

PhIP, like all HAAs, is a procarcinogen that requires metabolic activation. This activation generates DNA-binding species considered to be responsible for its carcinogenic potential. PhIP is primarily activated by hepatic CYP1A2-catalyzed N-hydroxylation and is detoxified by UGT1A-mediated glucuronidation [25]. To understand the effect of the diet types on PhIP activation and detoxification, we measured hepatic activities of CYP1A2 and UGT1A. There were statistically significant main effects of both diet ($P < 0.001$) and dietary PhIP concentration ($P < 0.0001$) for CYP1A2 activity, as well as a statistically significant interaction ($P = 0.009$) (Fig. 2A). That is, overall, there were differences in CYP1A2 activity due to diet, with groups fed the low-dose PhIP having greater CYP1A2 activity than those fed the high-dose PhIP. Among the low PhIP-fed groups, CYP1A2 activity was greater in the CRU group than in the basal (194%), TWD (143%), and API

groups (137%) ($P < 0.05$). The API group showed a trend towards greater CYP1A2 activity compared to the basal group (141%, $P = 0.06$). Among the high PhIP-fed groups, all TWD-based diets had equivalent CYP1A2 activity, which was greater than the basal group (150%, $P < 0.05$).

There was a trend towards a main effect due to diet in UGT1A activity ($P=0.097$), but no significant main effect due to dietary PhIP concentration and no interaction (Fig. 2B). UGT1A activity did not differ among the diet groups within either the low or the high PhIP-fed groups. However, there was a trend towards lower UGT1A activity in the high PhIP-fed API group compared to the high PhIP-fed TWD group (67%, $P = 0.071$).

### AC and ACF number

In this study, PhIP was administered in the diet, and therefore differences in food intake of the mice would alter the PhIP dose. Assuming a dose-dependent cancer risk, the ACF and AC numbers were normalized by food intake. There was a statistically significant main effect of both diet ($P = 0.031$) and dietary PhIP concentration ($P < 0.001$) on normalized ACF number, determined at the end of week 12, as well as a statistically significant interaction ($P = 0.0007$) (Fig. 3B). Overall, the high PhIP-fed groups had a greater number of ACF than the low PhIP-fed groups, regardless of diet. However, the effect of diet on ACF number was dramatically different depending on the dietary concentration of PhIP. In the low PhIP-fed groups, the TWD group had a greater number of ACF (123%, $P = 0.0053$) and AC (157%, $P = 0.0016$) compared to the basal diet, whereas both vegetable-containing TWDs had the effect on the formation of ACF similar to that of the basal diet. Unexpectedly, in the high PhIP-fed groups, the basal diet had a greater effect on the formation of ACF than
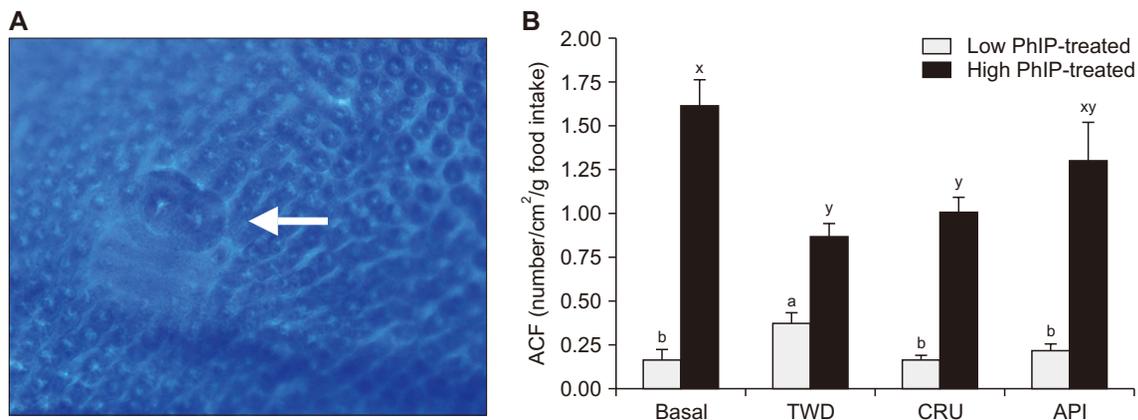


**Figure 3. The effect of CRUs and APIs on development of colonic ACF in mice fed PhIP as a carcinogen at two different dietary concentrations.** (A) Representative image of ACF in distal colon of A/J mice after staining with methylene blue (arrow; 40 × magnification). (B) Effect of TWD and API- or CRU-containing TWD on ACF in the low PhIP-treated groups (bright grey) and in the high PhIP-treated groups (dark grey). Values are presented as mean ± SEM; n = 8. Within a parameter, bars that do not share a letter are significantly different ($P < 0.05$) as determined by two-way ANOVA with the Duncan's post-hoc test. There were statistically significant main effects of diet ($P < 0.031$) and PhIP ($P < 0.001$) and a statistically significant interaction ($P = 0.0007$). ACF, aberrant crypt foci; TWD, total Western diet; CRU, cruciferous vegetable; API, apiaceous vegetable; PhIP, 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine.
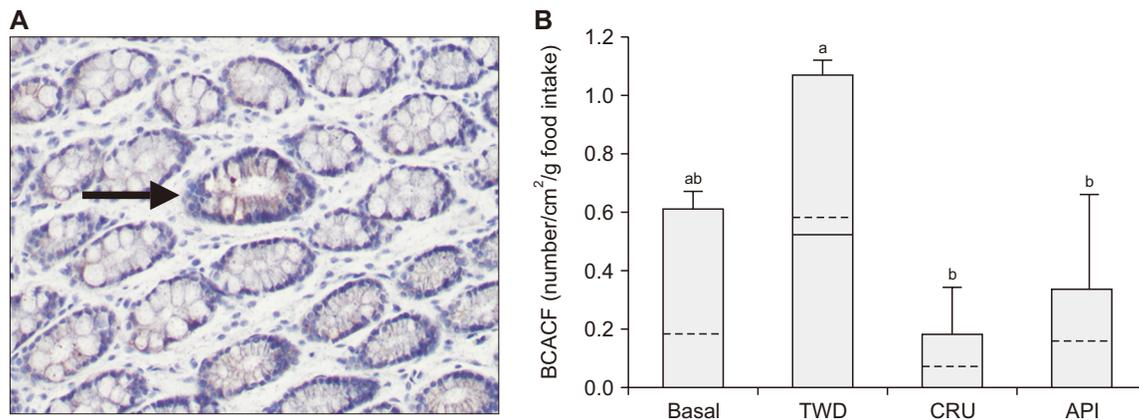
**Figure 4. Effects of CRU and API.** (A) Representative immunohistological image of BCACF in distal colons of A/J mice (arrow; 80 × magnification). (B) Effects of TWD and API- and CRU-containing TWD on BCACF in the low 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine-treated groups (bright grey). Box boundaries represent interquartile range of values, and error bars represent 5th %; n = 7-8. Values that do not share a letter are significantly different ($P < 0.05$). BCACF, β-catenin accumulated aberrant crypt foci; TWD, total Western diet; CRU, cruciferous vegetable; API, apiaceous vegetable.

the TWD (185%, $P < 0.05$). The CRU-containing TWD group had fewer ACF than the basal group (159%, $P < 0.05$) and an equivalent number of ACF to the TWD group. The ACF number in the API-containing TWD group was between the basal group and the TWD and CRU-containing TWD group and did not differ from either. Total AC number showed essentially the same pattern as ACF (data not shown). A representative image of an ACF is shown in Figure 3A.

### BCACF

Given that the reduction in AC and ACF of the vegetable-containing diet groups, relative to the TWD group, was only observed in low PhIP-fed groups, BCACF were determined in the colons of the low PhIP-fed groups. The number of BCACF did not differ between the basal diet group and the TWD group (Fig. 4B). Both the CRU and API groups had fewer BCACF compared to the TWD and basal groups ($P < 0.05$). A representative image of a BCACF is shown in Figure 4A.

### Correlations between morphological and immunohistochemical markers and biotransformation enzyme activities

Among all PhIP-treated groups, the ACF number and the hepatic CYP1A2 activity were strongly but inversely correlated ($r = -0.630$, $P < 0.0001$). However, neither the number of AC or ACF nor the activity of CYP1A2 correlated with UGT1A activity. Among low PhIP-treated groups, ACF number and BCACF number correlated modestly but statistically significantly ($r = 0.408$, $P = 0.025$). ACF number also correlated modestly with UGT1A activity ($r = 0.378$, $P = 0.047$), but not with CYP1A2 activity. There was no significant correlation between CYP1A2 activity and UGT1A activity. In high PhIP-treated groups, the number of ACF did not correlate with either CYP1A2 or UGT1A activity.
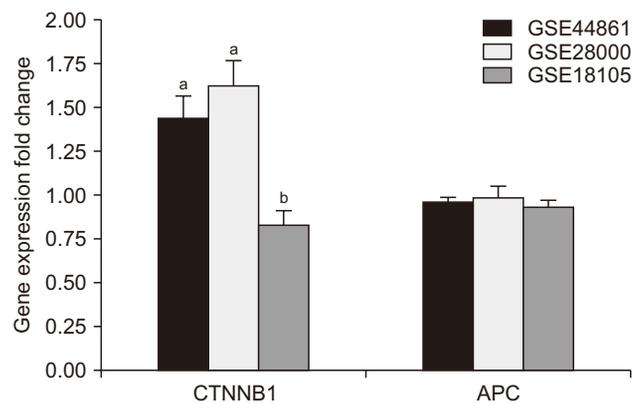


**Figure 5. Changes in colonic gene expression in cancerous vs. non-cancerous tissue in populations consuming a westernized diet or a diet high in vegetables.** Expression fold change of CTNNB1 and APC in colorectal adenocarcinomas compared to normal or noncancerous adjacent colorectal epithelium tissues from White only (GSE44861, n = 48), White and African American (GSE28000, n = 24), and Japanese (GSE18105, n = 17) subjects. Values are presented as mean ± SEM. Bars that do not share the same letter are significantly different ($P < 0.05$).

### Datamining clinical, public datasets to investigate the expression difference of CTNNB1 (β-catenin) and APC between American and Japanese colorectal adenocarcinoma

Western dietary patterns have been associated with greater risk of colon cancer, whereas diets categorized as healthy have been associated with a lower colon cancer risk [30]. The typical American diet is a WD that includes high consumption of red and processed meat, high-fat foods and dairy products, high-sugar desserts and drinks, as well as high intakes of refined grains [31]. Conversely, the Japanese dietary pattern is characterized by a diet rich in vegetables and other plant

foods [32], and therefore more closely fits the pattern of what is considered a healthy diet. Thus, comparing gene expression patterns between American and Japanese colon cancer patients may provide insight into the relevance of studies of differing dietary patterns on colon carcinogenesis in animal models. Therefore, we investigated gene expression changes in human colorectal AC using GEO colorectal AC data from two American-based cohort studies (GSE 28000 and GSE44861) and one Japanese-based cohort study (GSE 18105). β-Catenin gene expression was greater in colorectal AC vs. normal (adjacent noncancerous) colorectal epithelium tissues in both White and African American patients, whereas in Japanese patients, gene expression was less in adenocarcinomas than in normal tissue (Fig. 5). We also investigated the APC gene, upstream of β-catenin in WNT signaling and did not find significant difference between American and Japanese colorectal AC (Fig. 5).

## DISCUSSION

Epidemiological studies have consistently shown a positive association between a Western dietary pattern and colorectal cancer risk [2,3]. The TWD is a rodent diet that closely mimics the macronutrient and micronutrient composition of the average American diet [12]. By using a highly relevant background diet, the TWD, and a foodborne carcinogen, PhIP, the present study greatly reduces the translational gap between humans and experimental animals.

Mice differ in their susceptibility to carcinogen treatment. C57BL/6 mice appear resistant to the carcinogenic effect of PhIP, as these mice do not develop colonic tumors and few ACF when administered PhIP [33]. In contrast, A/J mice, used in the present study, are highly susceptible to carcinogen treatment [34]. Further, tumors from A/J mice show nuclear accumulation of β-catenin, indicating activation of the Wnt signaling pathway, frequently observed in human colonic tumors [35]. Thus, A/J mice appear to be an appropriate model for investigation of dietary influences on colon cancer risk induced by PhIP.

Based on an average daily energy intake of 10,878 kJ, the dietary concentration of PhIP would be approximately 0.043 ng/kJ. In the TWD containing the low dose of PhIP, the PhIP concentration would be a far greater amount of 5.33 µg/kJ, over 100,000 more concentrated. However, based on an average life expectancy of 78.8 years in Americans, the lifetime dietary exposure to PhIP in the US ranges from 9 mg to 18 mg. Using the high end of this range, and assuming an average body weight of 70 kg, this exposure is equivalent to 0.74 mg PhIP/kg metabolic body weight (MBW; $BW^{0.75}$). For our animal study, mean cumulative exposure to dietary PhIP was 7.1 mg for the low PhIP groups and 25.9 mg for the high PhIP groups. For mice consuming the low dose PhIP diets, this dose equates to 104 mg PhIP/kg MBW, or approximately 140 times greater than human exposure (0.74 mg PhIP/kg MBW).

For the high dose of PhIP, exposure was approximately 378 mg/kg MBW. Although few, if any, in the US population would have a PhIP exposure equivalent to that in this study, our PhIP exposures are consistent with the doses commonly used in rodent studies of carcinogenesis with a short duration of carcinogen exposure.

Among all diet groups, the number of ACF was considerably greater in animals consuming diets with the high dietary PhIP concentration (400 ppm) compared to the low dietary PhIP concentration (100 ppm). Among mice fed diets containing the low concentration of PhIP, those fed the TWD diet had significantly more ACF than those fed the basal diet. This is consistent with a recent report that A/J mice fed the TWD had significantly greater ACF number compared to a standard rodent diet when azoxymethane was used as the carcinogen [36]. One salient difference between the basal diet and the TWD is the higher fat content of the TWD. However, the effect of the dietary fat concentration on ACF development is inconsistent. Carcinogen-treated rats fed a high fat diet had a greater number of the ACF than rats fed a normal fat diet [37]. In contrast, carcinogen-treated CF1 mice fed a high fat diet showed no difference in ACF number compared to a normal fat basal group [38]. However, since the TWD differs from the basal diet in a number of important ways besides the fat content, including the fatty acid profile, concentrations of calcium and sodium, and vitamins D, E, and K, it may be more appropriate to consider the TWD in terms of a dietary pattern instead of individual nutrients. In this regard, it is of interest that a recent meta-analysis of dietary patterns and cancer risk reported a significant association between unhealthy diets (including red and processed meat, starchy foods and refined carbohydrates, and sugary drinks and salty snacks) and greater risk of colon cancer [5].

Surprisingly, in mice fed the high PhIP-containing diets, those consuming the TWD had significantly fewer ACF than those consuming the basal diet. Thus, the influence of the TWD on the formation of ACF number, relative to the basal diet, depended on the carcinogen dose. The reason for the opposite effect of the TWD on ACF development with different doses of PhIP is unclear. In contrast to the present study, in rats fed 400 ppm PhIP, those given a high fat, low calcium diet had a greater number of aberrant crypts than those fed a low fat, high calcium diet [39]. This raises the possibility that some other aspect of the TWD, besides its high fat and low calcium concentration, is responsible for the unexpected interaction between a carcinogen at different doses and westernized background diet. Alternatively, some aspect of the combination of high dietary PhIP and the TWD induces phase II enzymes involved in detoxification of PhIP, leading to a lesser amount of activated PhIP. This warrants further investigation and underscores the need for continued study of animal models of diet and carcinogenesis.

Epidemiological studies associate consumption of CRUs with reduced colon cancer risk [40], a correlation supported

by animal studies [23]. Recent findings in animals suggest that consumption of APIs may also reduce colon cancer risk [25]. However, whether these vegetables can reduce colon cancer risk in the context of a WD has not been previously examined. Our finding that in low PhIP-fed mice, both API- and CRU-containing TWDs resulted in fewer AC and ACF compared to animals fed TWD, and equivalent to those observed in mice fed the basal diet, indicates that these vegetables essentially counteract the increased risk of colon cancer induced by the TWD, and equivalently so. The effect of the vegetables on colon cancer risk when animals were fed the high dose of PhIP is less clear. Animals fed the high PhIP diet containing CRUs had significantly fewer ACF than the basal diet, but an ACF number equivalent to the TWD. Further studies of the influence of the carcinogen dose on the chemopreventive potential of dietary agents are warranted.

The Wnt/β-catenin signaling pathway is commonly dys-regulated in colorectal cancer [41]. β-Catenin functions as a structural protein at cell-cell adherens junctions and as a transcriptional activator in the Wnt signaling pathway [42]. β-Catenin accumulation in cytosol and nuclei occurs in a subset of ACF, which we refer to as BCACF, in rodent colons in response to carcinogen treatment [43]. BCACF are asso-ciated with a greater degree of dysplasia and greater risk for development of colon cancer [44]. Western-style dietary fat increased β-catenin accumulation in the colonic mucosa of rats treated with azoxymethane for 44 weeks [45]. Given the uncertain relevance of the high dietary concentration of PhIP used in the present study to the human situation, we enumer-ated BCACF only in groups fed the low dietary concentration of PhIP. The BCACF number did not differ significantly be-tween the TWD group and the basal diet group. Others, how-ever, have reported that carcinogen-treated rats fed a high fat cafeteria-style diet (i.e., a mix of highly palatable human foods) had greater ACF number and increased immunohisto-chemical expression of β-catenin in colon mucosa compared to a standard rodent diet [46]. Liu et al. [47] demonstrated that mice fed a very high fat diet (60% by energy) to induce obesity had an accumulation of β-catenin in their colonic mu-cosa, compared to mice fed a standard rodent diet. However, the TWD was found not to induce obesity in mice [48]. It is likely that obesity, and the chronic low level of inflammation that accompanies it [49], play an important role in colonic β-catenin accumulation.

Consumption of either CRUs or APIs incorporated into the TWD containing 100 ppm PhIP reduced the BCACF number relative to the TWD. Sulforaphane, an isothiocyanate derived from the glucosinolate glucoraphanin, abundant in brocco-li, decreased β-catenin accumulation, as well as reduced expression of cyclin D1, a Wnt/β-catenin target gene, in breast cancer cells [50]. 3,3'-Diindolylmethane, also derived from CRUs, reduced β-catenin accumulation in DLD-1 and HCT116 colon cancer cell lines [51]. To date, studies exam-ining the effect of bioactive compounds derived from APIs,

such as furanocoumarins or polyacetylenes, on β-catenin ac-cumulation are lacking. Our present study appears to be the first demonstration that CRUs and APIs reduce colonic β-cat-enin accumulation in a carcinogen-treated animal model.

Our findings appear to be consistent with those obtained in human populations with different dietary patterns. We used the GEO database (http://www.ncbi.nlm.nih.gov/geo/), which is a public repository of functional genomics data, to assess the potential clinical relevance of changes in β-catenin due to different dietary patterns. White American and African Amer-ican colorectal AC patients (GSE28000 [27] and GSE44861 [28]), who are more likely to consume a typical Western diet, have greater β-catenin gene expression in colorectal ade-nocarcinoma relative to normal colorectal epithelium tissues without overexpression of APC gene, whereas the opposite occurred in Japanese patients (GSE18105 [26]), who are more likely to consume a lower fat and higher vegetable-con-taining diet (Fig. 5). These results further support an associ-ation between dietary patterns and β-catenin expression in colon cancer.

Modifications in PhIP metabolism, due to altered activity or expression of enzymes involved in its detoxification and/ or activation, may change its carcinogenic potential. CYP1A2 is the enzyme primarily responsible for the initial hydroxyl-ation of PhIP, leading eventually to an active carcinogen [52]. It has been reported that PhIP induces its own metabolism [53]. However, in the present study the inverse was true, as the high dose of PhIP had a much lower activity of CYP1A2 activity than the low dose, yet animals fed the high PhIP di-ets had greater numbers of ACF. The reason for this inverse relationship between the dietary PhIP concentration and hepatic CYP1A2 activity is unknown and does not appear to have been previously reported. However, since hepatic UGT1A activity, which is responsible for the detoxification of PhIP by glucuronidation [25], was unchanged by the PhIP diet concentration, it seems likely that even with the reduction in CYP1A2 activity, the higher dietary concentration of PhIP resulted in a greater amount of PhIP being activated, leading to a greater number of ACF in the groups fed the high dietary concentration of PhIP.

In the high PhIP-containing diets, the overall lower number of ACF in the TWD-based diets, compared to the basal diet, suggests that, at a high dietary concentration of PhIP, some component of the TWD alters carcinogen metabolism, such that less active carcinogen is formed. All TWD-based diets significantly increased CYP1A2 activity relative to the basal diet by approximately 150%. The basal and TWD diets do not differ from one another in terms of known inducers or inhibitors of CYP1A2 activity [54]. However, the TWD has a greater dietary fat concentration than the basal diet and con-tains cholesterol. Since mice fed a high fat high cholesterol diet were shown to have greater hepatic gene expression of CYP1A2 than mice fed a normal fat cholesterol free diet [55], the greater fat and cholesterol content of the TWD diet may

be responsible for the increased CYP1A2 activity in mice fed the TWD, compared to the basal diet. The activity of UGT1A did not differ between the TWD and basal diets, suggesting that the TWD did not influence PhIP detoxification by glucuronidation.

Mice fed the low PhIP-containing CRU diet had greater CYP1A2 activity than all other diets, consistent with our previous finding that consumption of CRUs increases hepatic CYP1A2 activity in rats [56]. This is paradoxical, given that the low PhIP-containing CRU diet group had significantly fewer ACF than the low PhIP-containing TWD group and that, in a previous study, animals administered PhIP and fed a diet containing CRUs showed a trend towards fewer PhIP-DNA adducts in the colon [25]. One possible explanation for this apparent contradiction involves the transcription factor p53, which is activated in response to DNA damage, and plays a role in many metabolic processes related to cell survival or death. In mice administered PhIP, there were more DNA adducts in p53 knockout mice than in wild-type mice in both liver and colon [57]. In rats given sinigrin, a glucosinolate derived from CRUs, hepatic gene expression of p53 was increased [58]. Similarly, in the HEp-2 human epithelial carcinoma cell line, incubation with the glucosinolate sulforaphane resulted in increased p53 [59]. Thus, consumption of CRUs may stimulate p53 synthesis, thereby reducing DNA adducts, which leads to formation of fewer ACF.

In summary, we found that a rodent diet (TWD) mimicking the dietary pattern of typical Americans increased the formation of ACF, a marker of colon cancer risk, in mice administered the foodborne carcinogen PhIP at a dietary concentration of 100 ppm. When the TWD included mixtures of either CRUs or APIs, the ACF-promoting effect of the TWD was eliminated. However, when PhIP was fed at a dietary concentration of 400 ppm, the number of ACF was lower in the TWD diet relative to the basal diet, and a chemopreventive effect of the vegetables was not evident. Thus, a high dose of carcinogen may mask the effects of chemopreventive agents. This should be considered when selecting an optimal carcinogen dose in chemoprevention studies.

## ACKNOWLEDGMENTS

## CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

## SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.2020.25.4.223.

## DATA AVAILABILITY

The Gene Expression Omnibus (GEO) datasets used in the current study are available from the GEO database with accession numbers GSE18105, GSE28000 and GSE44861.

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