Subjects were asked to sit calmly and without talking for at least 2 min before measurement, with their legs uncrossed and their arms crossed at heart level.

Medical history, smoking status and anthropometric data were also collected at the annual health check-up. Classification as hypertensive or normotensive was based on the results of the health check-up. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared.

Outcomes and statistical analysis

The effects of dietary intervention were examined using data from the first half of the study; that is, the group receiving dietary intervention in the first year as the intervention group. Because follow-up study after termination of the intervention suggested that the effects of intervention on diet were maintained well over 4 years, the decision was made to simplify assessment by excluding the data from the second half of the study. Subjects were excluded from the analysis of dietary data if they met either of the following criteria: (1) the DHQ was incomplete for either the pre- (baseline) or post-intervention (year 1); and (2) estimated energy intake was less than 50% of the energy requirement for a sedentary lifestyle or greater than 150% of that for a vigorous lifestyle.

Primary study outcome was the effect of intervention during the first year, namely the difference in changes between the intervention and control groups. Mean daily intakes of energy, targeted nutrients and mean urinary sodium at baseline and year 1 were calculated, with values at each point for fruits and vegetables, as well as alcohol, carotene, and vitamin C, transformed by the natural logarithm before calculation, to account for the skewing of distribution to the right. Mean values of variables for the groups at baseline were compared by the *t*-test. Proportions at baseline were tested by the χ^2 test. Differences from baseline to year 1 within groups are presented with 95% confidence intervals (95% CI). Analyses of covariance (ANCOVA) were conducted to investigate differences in outcome measure at year 1 between the randomized groups. Baseline values of each variable were included as covariates. For BP analysis, baseline BP and change in alcohol intake and body weight were included as covariates. All analyses were done with SAS statistical software (SAS Institute Inc., Cary, North Carolina, USA, version 8.0).

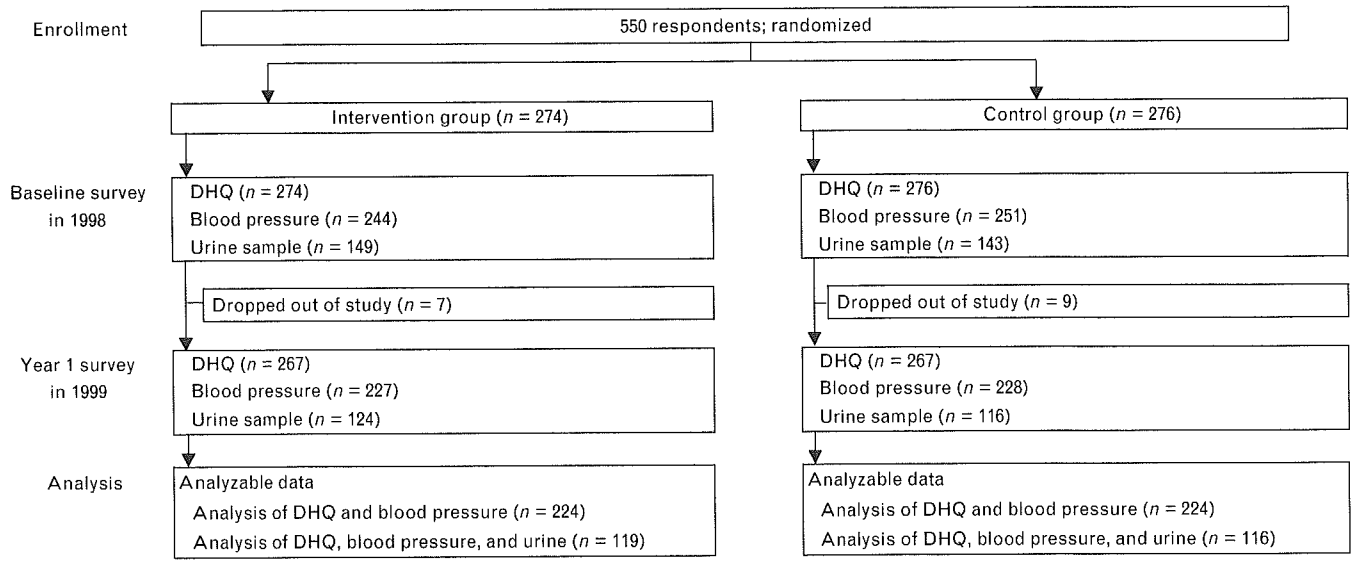
Results

Baseline characteristics

A total of 292 urine samples were obtained from 550 respondents who completed the DHQ at baseline (Fig. 1), and 240 samples from 534 subjects who completed the DHQ at year 1. Analysis included 235 and 448 subjects with and without urinary data, respectively.

Table 1 shows baseline variables for the intervention and control groups. Mean age of participants was 56.4 years. Mean systolic BP (SBP) and diastolic BP (DBP) did not statistically differ between the groups. Among participants, 11% (26 and 23 subjects in the intervention and control group, respectively) were receiving antihypertensive drug treatment at the beginning of the trial. Antihypertensive medication status of these subjects

Fig. 1



Number of study subjects. DHQ, diet history questionnaire.

Table 1 Subject characteristics at baseline

	Intervention group ($n = 224$)	Control group ($n = 224$)	P^a
Age (years)[b]	56.3 (41.2, 71.4)	56.4 (40.5, 72.4)	0.863
Sex (% female)	68.3	67.0	0.762
Body height (cm)[b]	154.9 (140.6, 169.2)	155.2 (139.1, 171.4)	0.978
Body weight (kg)[b]	56.7 (39.5, 73.8)	56.0 (39.1, 72.9)	0.996
Body mass index (kg/m^2)[b]	23.6 (17.9, 29.3)	23.2 (17.6, 28.8)	0.949
Blood pressure (mmHg)[b]			
Systolic blood pressure	127.9 (93.2, 162.5)	128.0 (97.4, 158.5)	0.955
Diastolic blood pressure	75.9 (53.9, 97.8)	76.3 (55.9, 96.6)	0.720
Alcohol drinker (%)	38.8	40.2	0.772
Hypertension (%)	23.7	24.1	0.912
On antihypertensives (%)	11.6	10.3	0.650
Diabetes (%)	3.6	3.1	0.793
Hyperlipidemia (%)	5.8	10.3	0.082

[a]P value for comparison between groups. [b]Values are mean and 95% confidence intervals.

did not change throughout the trial (data not shown). There were no statistically significant differences between the groups in age and sex distribution or other baseline variables.

Effect of intervention on lifestyle factors and dietary variables

Mean body weight and lifestyle factors and their changes from baseline to year 1 are presented in Table 2. No statistically significant change was observed between the groups. Daily intake of energy and nutrients and their changes are also presented in Table 2. At year 1, intake of fruit and vegetables and of dietary carotene and vitamin C increased significantly more in the intervention group ($P < 0.05$). Sodium intake in the intervention group decreased by 15 mmol/day (95% CI: −26, −4), but increased by 11 mmol/day (−0, +22) in the control group. This difference in change between the two groups was statistically significant ($P = 0.002$). Mean urinary excretion of sodium and potassium and the corresponding daily intake are shown in Table 3. Excretion and intake of sodium in the intervention group decreased by 49 (95% CI: −62, −36) and 11 mmol/day (−25, +4), respectively. This difference in change between the two groups was statistically significant ($P < 0.001$).

Effects of intervention on BP

Table 4 shows that SBP in the intervention group decreased from 127.9 to 125.2 mmHg (−2.7 mmHg change; 95% CI: −4.6, −0.8), but in the control group increased from 128.0 to 128.5 mmHg (+0.5 mmHg change; −1.3, +2.3), with this difference in change between the groups being statistically significant ($P < 0.01$). DBP changed from 75.9 to 74.8 mmHg (−1.0 mmHg change; 95% CI: −2.4, +0.3) in the intervention and from 76.3 to 75.9 mmHg (−0.3 mmHg change; −1.7, +1.1) in the control group. This difference in change between the groups was not statistically significant.

Data for the subgroup of subjects from whom urine was collected were analyzed separately. Results showed no difference in baseline SBP and DBP between those with and without urine collection (data not shown). SBP changed for urine collectors by −3.0 mmHg (95% CI: −5.7, −0.2) in the intervention group, whereas it changed by +0.3 mmHg (−2.5, +3.1) in the control group. This difference in change between the groups being statistically significant ($P < 0.05$).

BP data were also analyzed by hypertensive status. In the hypertensive subjects, SBP changed by −5.6 mmHg (95% CI: −9.3, −2.0) in the intervention group, whereas it changed by +1.4 mmHg (95% CI: −3.6, 6.3) in the control group. This difference in change between the groups was statistically significant ($P < 0.05$). In normotensive subjects, the decrease in SBP was also greater in the intervention group compared to the control group, but this difference in change did not reach the level of statistical significance ($P = 0.075$). Further, no statistically significant changes in DBP were observed between the two groups in either subgroup analysis.

Discussion

This 1-year dietary, moderate-intensity, community-based intervention trial demonstrated a significant decrease in SBP level. A greater decrease in average dietary intake and urinary excretion of sodium was seen in the intervention group than in the control group. An increase in fruit and vegetable intake was accompanied by an increase in carotene and vitamin C intake.

Several community-based, large-scale, randomized trials on the effects of dietary intervention on BP have been reported to date. However, the variability of lifestyle intervention topics and their intensity makes it difficult to compare the BP changes achieved. Further, most of these previous studies targeted only hypertensive subjects.

Changes in BP and urinary sodium

Brunner *et al.* [14] performed a meta-analysis of randomized controlled trials designed to investigate the primary prevention of chronic diseases, and evaluated the effects

Table 2 Body weight and nutrient and food intakes at each point

	Intervention group ($n = 224$)			Control group (n = 224)				
	Baseline Mean (95% CI)	Year 1 Mean (95% CI)	Change[c] (95% CI)	Baseline Mean (95% CI)	Year 1 Mean (95% CI)	Change[c] (95% CI)	Adjusted between-group difference in change[d] (95% CI)	Adjusted *P* value[e]
Body weight (kg)	56.7 (39.5, 73.8)	56.5 (39.4, 73.7)	−0.1 (−0.3, 0.1)	56.0 (39.1, 72.9)	55.9 (39.2, 72.7)	−0.1 (−0.4, 0.1)	0.0 (−0.3, 0.3)	0.907
Moderate physical activity[a] no. (%)	223 (99.6%)	223 (99.6%)	0 (0%)	224 (100%)	224 (100%)	0 (0%)	0 (0%)	–
Current smoker[a] no. (%)	22 (9.8%)	22 (9.8%)	0 (0%)	26 (11.6%)	26 (11.6%)	0 (0%)	0 (0%)	–
Energy intake (MJ/day)	8.56 (3.43, 13.68)	8.44 (3.46, 13.41)	−0.12 (−0.38, 0.15)	8.15 (3.36, 12.96)	8.30 (3.40, 13.20)	0.16 (−0.13, 0.44)	−0.13 (−0.49, 0.22)	0.454
Alcohol (g/day)	3.5[b] (−1.6, 76.4)	3.0[b] (−1.6, 57.7)	−2.9 (−4.7, −1.2)	3.6[b] (−1.6, 76.2)	3.2[b] (−1.6, 65.7)	−1.6 (−3.7, 0.5)	−1.3 (−3.6, 1.0)	0.270
Nutrient intake								
Carotene (μg/day)	2159[b] (507, 9196)	2622[b] (677, 10156)	468 (166, 771)	1789[b] (331, 9676)	1944[b] (377, 10036)	170 (−77, 418)	521 (184, 858)	0.003
Vitamin C (mg/day)	107[b] (32, 358)	123[b] (42, 362)	15 (3, 26)	95[b] (24, 376)	100[b] (27, 364)	1 (−9, 12)	19 (6, 31)	0.003
Sodium (mmol/day)	237 (76, 397)	222 (54, 390)	−15 (−26, −4)	229 (64, 395)	240 (61, 420)	11 (−0, 22)	−23 (−37, −8)	0.002
Potassium (mmol/day)	71 (18, 123)	73 (18, 128)	2 (−1, 6)	65 (17, 113)	66 (20, 113)	1 (−2, 4)	4 (−1, 8)	0.081
Dietary fiber (g/day)	15.6 (3.1, 28.0)	16.2 (4.1, 28.3)	0.6 (−0.2, 1.5)	14.3 (3.2, 25.4)	14.6 (4.2, 25.1)	0.3 (−0.4, 1.0)	1.0 (0.0, 1.9)	0.040
Calcium (mg/day)	691 (59, 1323)	690 (106, 1274)	−1 (−45, 43)	621 (129, 1113)	663 (83, 1244)	42 (6, 78)	−7 (−56, 43)	0.792
Food intake								
Vegetables (g/day)	252.8[b] (72.2, 879.3)	269.3[b] (85.0, 848.1)	15.1 (−12.8, 42.9)	226.4[b] (62.4, 815.2)	227.3[b] (65.7, 781.0)	−2.1 (−19.7, 15.5)	34.2 (5.3, 63.0)	0.020
Fruits (g/day)	63.3[b] (7.5, 486.9)	84.5[b] (12.4, 543.0)	24.3 (11.5, 37.1)	59.3[b] (6.2, 503.5)	63.1[b] (6.8, 523.8)	2.0 (−10.0, 13.9)	23.1 (7.9, 38.4)	0.003

[a]Number and percentage of subjects. [b]Mean values at each point were transformed by the natural logarithm before computation because of the skewed distributions. They were back-transformed to show means and 95% confidence intervals (CIs). [c]Difference between baseline and year 1. [d]Difference between intervention group and control group in change after adjustment for baseline intake. [e]*P* values for comparison of mean at year 1 between the intervention group and control group by ANCOVA after adjustment for baseline intake.

Table 3 Dietary intake and 48-h urinary excretion of sodium and potassium [means and 95% confidence intervals (CIs)] among subjects who completed urine collection at two points

	Intervention group ($n = 119$)			Control group ($n = 116$)				
	Baseline Mean (95% CI)	Year 1 Mean (95% CI)	Change[b] (95% CI)	Baseline Mean (95% CI)	Year 1 Mean (95% CI)	Change[b] (95% CI)	Adjusted between-group difference in change[c] (95% CI)	Adjusted *P* value[d]
Sodium (mmol/day)								
Dietary intake	242 (85, 398)	229 (65, 393)	−13 (−29, 3)	235 (78, 392)	247 (65, 428)	12 (−5, 28)	−21 (−41, −1)	0.040
Urinary excretion[a]	248 (103, 393)	199 (62, 335)	−49 (−62, −36)	248 (94, 402)	237 (50, 424)	−11 (−25, 4)	−39 (−56, −21)	<0.001
Potassium (mmol/day)								
Dietary intake	73 (22, 123)	75 (25, 125)	2 (−3, 7)	69 (20, 117)	69 (22, 117)	1 (−4, 5)	4 (−2, 9)	0.204
Urinary excretion[a]	66 (26, 107)	59 (18, 100)	−7 (−11, −3)	66 (27, 105)	61 (17, 105)	−4 (−8, −1)	−2 (−7, 3)	0.408

[a]Expected intake was considered to be observed urinary excretion divided by 0.86 for sodium and 0.77 for potassium. See text for details. [b]Difference between baseline and year 1. [c]Difference between intervention group and control group in change after adjustment for baseline value. [d]*P* values for comparison of mean at year1 between the intervention group and control group by ANCOVA after adjustment for baseline value.

Table 4 Blood pressure [mean and 95% confidence interval (CI)] at two points

	Intervention group			Control group				
	Baseline Mean (95% CI)	Year 1 Mean (95% CI)	Change[a] (95% CI)	Baseline Mean (95% CI)	Year 1 Mean (95% CI)	Change[a] (95% CI)	Adjusted between-group difference in change[b] (95% CI)	Adjusted *P* value[c]
All subjects		($n = 224$)			($n = 224$)			
SBP (mmHg)	127.9 (93.2, 162.5)	125.2 (93.7, 156.7)	−2.7 (−4.6, −0.8)	128.0 (97.4, 158.5)	128.5 (99.0, 158.0)	0.5 (−1.3, 2.3)	−3.1 (−5.4, −0.9)	0.007
DBP (mmHg)	75.9 (53.9, 97.8)	74.8 (53.3, 96.4)	−1.0 (−2.4, 0.3)	76.3 (55.9, 96.6)	75.9 (55.9, 95.9)	−0.3 (−1.7, 1.1)	−0.9 (−2.6, 0.8)	0.307
Urine collection		($n = 119$)			($n = 116$)			
SBP (mmHg)	128.0 (91.6, 164.3)	125.0 (92.9, 157.2)	−3.0 (−5.7, −0.2)	128.4 (98.6, 158.2)	128.7 (98.1, 159.3)	0.3 (−2.5, 3.1)	−3.4 (−6.8, −0.0)	0.048
DBP (mmHg)	75.8 (53.5, 98.2)	74.5 (53.8, 95.2)	−1.3 (−3.1, 0.4)	76.8 (57.6, 96.1)	75.8 (56.5, 95.0)	−1.1 (−3.0, 0.9)	−0.8 (−3.0, 1.5)	0.502
Normotensive		($n = 171$)			($n = 170$)			
SBP (mmHg)	123.3 (91.2, 155.4)	121.6 (91.8, 151.3)	−1.8 (−4.0, 0.4)	124.1 (98.2, 150.0)	124.3 (98.3, 150.3)	0.2 (−1.6, 2.1)	−2.3 (−4.7, 0.2)	0.075
DBP (mmHg)	73.6 (52.6, 94.5)	72.5 (52.6, 92.5)	−1.1 (−2.7, 0.5)	74.7 (55.6, 93.8)	74.2 (54.8, 93.6)	−0.5 (−2.1, 1.1)	−1.2 (−3.1, 0.7)	0.233
Hypertensive		($n = 53$)			($n = 54$)			
SBP (mmHg)	142.5 (116.2, 168.8)	136.9 (111.4, 162.3)	−5.6 (−9.3, −2.0)	140.2 (108.4, 172.0)	141.6 (116.6, 166.5)	1.4 (−3.6, 6.3)	−5.2 (−9.9, −0.4)	0.032
DBP (mmHg)	83.3 (64.5, 102.0)	82.4 (62.5, 102.3)	−0.9 (−3.3, 1.5)	81.1 (59.9, 102.3)	81.3 (63.0, 99.6)	0.2 (−2.9, 3.3)	0.1 (−3.3, 3.4)	0.971

[a]Difference between baseline and year 1. [b]Difference between intervention group and control group in change after adjustment for baseline values and change in alcohol intake and body weight. [c]*P* values for comparison of mean at year 1 between the intervention group and control group by ANCOVA after adjustment for baseline values and changes in alcohol intake and body weight. SBP, systolic blood pressure; DBP, diastolic blood pressure.

of dietary change on BP in free-living subjects. This meta-analysis included relatively intensive dietary interventions such as monthly group sessions and several individual counseling sessions. Among the four studies which aimed to reduce sodium intake, overall mean net urinary sodium reduction was 32 mmol/24 h. Further, the mean net BP changes over 9–18 months were −1.9 mmHg (95% CI: −3.0, −0.8) for SBP and −1.2 mmHg (−2.6, 0.2) for DBP. The net changes in BP and urinary sodium excretion seen in the present study were slightly greater than the results of this meta-analysis.

Change in BP among hypertensive subjects

Analysis of subjects by hypertensive status showed that the effect of dietary modification on BP was greater in subjects of the hypertensive subgroup. The most recent meta-analysis of clinical trials of salt reduction [15] showed a 4.96/2.73 mmHg decrease in hypertensives ($P < 0.001$ for both SBP and DBP). The effect on SBP in the present hypertensive subjects was comparable with these previous results. Dietary modification might prevent or delay the initiation of medication in hypertensive subjects with BP levels that straddle the threshold for antihypertensive medication.

Changes in BP and intake of other nutrients and lifestyle factors

The present study focused on the increase in the intake of vitamin C and carotene by recommendation of more fruits and vegetables. Results showed a moderate increase in carotene, vitamin C and dietary fiber intake, but not in potassium or calcium. Previous observational studies have reported significant inverse associations between BP and the intake of vitamin C, dietary fiber, potassium, magnesium and calcium [16–19]. The effectiveness of these nutrients has also been confirmed in clinical trials of dietary fiber, potassium, magnesium and calcium [20–23]. Furthermore, lifestyle factors such as physical activity, weight loss, alcohol consumption and smoking also influence BP level [24–26]. We did not observe changes in these variables. The decrease in BP seen in the present study might be attributable at least to some extent to increases in the intake of vitamin C and dietary fiber.

Changes in BP and fruit and vegetable intake

The present study focused on the use of fruits and vegetables to increase carotene and vitamin C intake.

Several previous clinical studies have examined the effect of dietary intervention on BP. The DASH trial, a well-controlled, randomized, clinical trial [7] to assess the effects of dietary patterns on BP, showed a decrease in SBP of 2.8 mmHg and in DBP by 1.1 mmHg by an increase in dietary fruit and vegetable intake for 8 weeks. In a subsequent study [27], this hypotensive effect of the

DASH diet was enhanced by its combination with a reduced sodium diet (100 and 50 mmol/day). The results of the present study support those of the DASH trials by showing similar, albeit somewhat weaker, results in a free-living, general population.

Further, Nowson *et al.* [28] conducted a cross-over dietary intervention study using a community-living subject. They reported a significant decrease in BP by a low-sodium, high-potassium diet and a DASH-type diet (DASH diet with moderate sodium reduction). The tendency of the results was similar to those of the present study, but the size of the effect was greater. However, the study population in their study was smaller ($n = 94$), the study period shorter (4 weeks), and the intervention more intensive (bi-weekly contact) than in the present study. Salt-free bread, salt-free margarine or both were provided to the intervention subjects. In contrast, no food was provided in the present study. The method used in the present study appears to be more practicable for use in community settings than that in the study of Nowson *et al.* [28].

Study limitations

Because this study was an open trial, the possibility of interaction between the intervention and control groups, such as information exchange, cannot be ruled out. In the control group, however, no statistically significant change in targeted nutrients and foods between the baseline and year 1 points was observed, suggesting that any interaction between the groups may have been negligible. Nevertheless, the possibility of some general information exchange remains, and the results should therefore be interpreted with caution.

To examine a practical model for population-based lifestyle improvement intervention, our present intervention study was performed in a primary health care setting rather than an academic center setting. Measurement of BP was conducted at the annual health check-up as a routine component of that check-up, and thus only a single measurement was done instead of multiple measurement. Nevertheless, conditions between the two groups were the same.

Because they had been previously exposed to various public health campaigns in the study area on the importance of decreasing salt intake, the present study subjects were relatively well-motivated to reduce their salt intake [29]. Moreover, they were provided further information about the unfavorable effect of dietary sodium on health prior to the start of the study. We therefore presume that they were more receptive to the message to decrease sodium given in this study. The decrease in sodium and consequent decrease in BP observed in this trial indicates the effectiveness of this intervention method for motivated persons. Further studies are necessary to determine whether this intervention method is equally effective on dietary and BP modification in other populations.

In conclusion, these findings indicate that the effects of dietary interventions undertaken to reduce the intake of sodium and increase that of fruit and vegetables may be expected to decrease BP level in free-living populations. The present randomized, controlled trial involved a relatively large number of free-living subjects and examined the change in dietary habits and BP over 1 year. The intervention method used here may represent an efficient and practicable model for population-based BP improvement in common primary care settings.

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Folate, Vitamin B_6, Vitamin B_{12}, and Vitamin B_2 Intake, Genetic Polymorphisms of Related Enzymes, and Risk of Colorectal Cancer in a Hospital-Based Case-Control Study in Japan

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Abstract: *We conducted a case-control study to investigate the association of nutrient intake involved in the one-carbon pathway of folate for DNA methylation and DNA synthesis and the related enzyme genetic polymorphisms with colorectal cancer. Cases were 107 patients newly diagnosed with colorectal cancer. Controls were 224 subjects matched with cases by sex, age, and residential area. Nutrient intake was assessed by a self-administered, semiquantitative food-frequency questionnaire. Four genetic polymorphisms—*MTHFR *C677T and A1298C,* MTRR *A66G, and* ALDH2 *Glu487Lys—were determined using blood samples. Odds ratios were calculated using conditional logistic regression analysis adjusted for smoking, alcohol consumption, body mass index, and dietary fiber intake. Although folate intake was inversely associated with colorectal cancer, this association was attenuated after further controlling for dietary fiber intake. Neither vitamin B_6, vitamin B_{12}, nor vitamin B_2, nor any genetic polymorphism was significantly associated with colorectal cancer.* MTRR *polymorphism interacted with the association of folate (*P *for interaction = 0.04) or vitamin B_6 (*P *for interaction = 0.02) with colorectal cancer, although the other polymorphisms did not interact with any nutrient intake. In conclusion, the study did not support the existing hypothesis of gene–nutrient interaction in colorectal carcinogenesis.*

Introduction

Folate provides the one-carbon groups in the synthesis of thymidilates and the methylation of DNA and protein (1,2). Folate deficiency is assumed to cause uracil misincorporation, leading to DNA instability (3), and a retarded DNA repair for oxidative or alkylating damage, which has been implicated in the development of cancer (4). Such folate insufficiency can also lead to global (5) and proto-oncogenic DNA hypomethylation (6), resulting in human carcinogenesis including the large bowel.

Vitamins B_6 and B_{12} are involved in this folate metabolism pathway (2,7). Vitamin B_6 works as a cofactor for serine hydroxymethyltransferase, which catalyzes the formation of glycine and 5,10-methylenetetrahydrofolate from serine and tetrahydrofolate. This is an enzyme related to folate metabolism. Moreover, vitamin B_6 works as a cofactor for cystathionine-β-synthase, which catalyzes the conversion of homocysteine to cystathionine, a pathway that competes with the remethylation of homocysteine by methionine synthase to methionine. Vitamin B_{12} is a cofactor of methionine synthase and is important for maintaining adequate intracellular levels of methionine.

Vitamin B_2 (riboflavin) is the precursor for flavin adenine dinucleotide, the cofactor for methylenetetrahydrofolate reductase (*MTHFR*). The combination of vitamin B_2 intake and the genetic polymorphism C677T of *MTHFR* possibly affects folate metabolism, leading to colorectal carcinogenesis (8,9).

MTHFR metabolizes 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate and provides methyl groups to DNA methylation via the remethylation of homocysteine. This enzyme has a genetic polymorphism of C to T in the 677th base pair that causes a substitution of codon 222 alanine to valine and leads to lower activity of the enzyme than with no T variant (10). Previous studies revealed that the TT genotype (11,12) or the TT genotype with higher plasma folate (13,14) was inversely associated with colorectal cancer.

MTHFR has another common genetic polymorphism of A to C in the 1,298th base pair that causes a substitution of codon 429 glutamate to alanine. This polymorphism also

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leads to lower activity of the enzyme (15). Although this polymorphism was associated with a decreased risk of colorectal cancer (12), the evidence on the interaction with folate is sparse and inconsistent (16–19).

Methionine synthase reductase (*MTRR*) maintains methionine synthase in an active form. The methionine synthase is dependent on vitamin B_{12}. *MTRR* also has a common genetic polymorphism of A to G in the 66th base pair that causes a substitution of codon 22 isoleucine to methionine (20). This polymorphism may modify the effect of folate, vitamin B_6, or vitamin B_{12} to colorectal cancer (16), although the function of the polymorphism is still unknown.

Alcohol consumption was associated with folate malabsorption and folate deficiency (21,22). Folate deficiency was associated with a more-increased risk of colorectal cancer in drinkers (23–26), and drinkers were not associated with the decreased risk of colorectal cancer even if they had a high blood level of folate (14). Aldehyde dehydrogenase 2 (*ALDH2*) is one of the key enzymes of alcohol metabolism, and its genetic polymorphism of codon 487 glutamate to lysine broadly exists in Orientals including Japanese (27). Such a polymorphism exerts low activity of *ALDH2* (28) and causes high blood levels of acetaldehyde (29). The acetaldehyde possibly induces cleavage of folate (22,30). Therefore, the *ALDH2* genetic polymorphism may interact with folate in relation to colorectal cancer. However, to our knowledge, such interaction has never been investigated.

The folate and *MTHFR* C677T polymorphism interaction with colorectal cancer has been examined quite extensively, and evidence has been accumulated (12,31). In particular, evidence on the interaction between plasma folate and this polymorphism (13,14) has been accumulated. However, the interaction between other nutrients including vitamin B_6, vitamin B_{12}, and vitamin B_2 and other related enzyme genetic polymorphisms such as *MTHFR* A1298C (16–19), *MTRR* A66G (16), and *ALDH2* Glu487Lys has not yet been sufficiently investigated. These single nucleotide polymorphisms (SNPs) were selected for investigation because they related to the metabolism of folate and alcohol and were nonsynonymous SNPs in the coding region.

We investigated the association of folate, vitamin B_6, vitamin B_{12}, vitamin B_2, and genetic polymorphisms of related enzymes with colorectal cancer and also examined the interaction between these nutrients and genetic polymorphisms in relation to colorectal cancer risk in a multicenter, hospital-based case-control study.

Materials and Methods

Study Subjects

A hospital-based case-control study of gastrointestinal cancer was conducted between October 1998 and March 2002 at four hospitals in Nagano Prefecture, Japan (32,33). Eligible cases were colorectal cancer patients aged 20–74 yr who had been newly diagnosed during the survey at those hospitals. Consequently, we collected 121 colorectal cancer cases. No patient refused to participate in our study. We selected controls from medical checkup examinees in the four hospitals. Eligible healthy controls were those who were confirmed to have no cancer by the medical checkup, which included upper gastrointestinal endoscopy or X-ray, fecal occult blood test, or abdominal ultrasound, and, if screened in this medical checkup, confirmed by a subsequent detailed checkup. Two controls were matched for each case by sex, age (within 3 yr), and residential area during the study period in the same hospitals. Some cases had only one or more than two controls by way of exception. Although 249 potential controls were selected, 2 duplicate subjects enrolled were excluded. Of 247 controls, 2 individuals refused to participate in this study. Thus, 245 controls participated (participation rate = 99%) as matched controls. All cases were histopathologically confirmed according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (34). We obtained written informed consent from all cases and controls. This study was approved by the Institutional Review Board of the National Cancer Center, Tokyo, Japan.

Exposure Assessment

We asked study subjects to answer a self-administered questionnaire that included general characteristics, such as age, sex, occupation, personal medical history, family history of disease, including colorectal adenoma and cancer, smoking and drinking habits, vitamin supplement use, and dietary habits. Their habitual consumption of foods and beverages was assessed with a 141-item, semiquantitative food-frequency questionnaire (FFQ). Subjects reported their average frequency of consumption and average portion size for those items during the past year. If subjects had any present symptoms, they provided their dietary habits for the year before the onset of symptoms. The mean daily consumption of energy and nutrients including folate, vitamin B_6, vitamin B_{12}, and vitamin B_2 was calculated using the food composition table developed for this FFQ based on the *Standard Tables of Food Composition in Japan*, 5th revised edition (35,36). The most important dietary sources were rice, spinach, and green tea for folate; rice, tuna, potatoes, bananas, and beer (only in men) for vitamin B_6; pacific saury, salted salmon roe, squid, mackerel, and clam for vitamin B_{12}; and egg, milk, rice, and green tea for vitamin B_2. Vitamin supplements were not included in the nutrient intake estimation because of no detailed information on B vitamins. Vitamin-enriched rice was included to calculate vitamin B_2 intake. The estimated consumption was validated with 14- or 28-day dietary records (DRs) and biomarkers in a prior study (37). The deattenuated rank correlation coefficients between FFQ and DR were as follows: 0.57 for folate, 0.59 for vitamin B_6, and 0.52 for vitamin B_{12} in men; 0.47 for folate, 0.63 for vitamin B_6, and 0.58 for vitamin B_{12} in women (unpublished data). The validity as rank correlation of vitamin B_2 intake was 0.34 in men and 0.45 in women (38).

Genotyping

Blood samples from subjects were collected at the same time as the questionnaire, and a buffy coat was preserved at –80°C until analysis. We determined four single-nucleotide polymorphisms (SNPs) in the following three genes: *MTHFR*, *MTRR*, and *ALDH2*. We used the MassARRAY (39,40) to measure SNPs in *MTHFR* and *ALDH2* and used the TaqMan® SNP Genotyping Assay of Applied Biosystems to measure an SNP in *MTRR* (Assay ID, C-3068176-10; dbSNP ID, rs1801394) (41). All four SNPs caused amino acid substitutions: codon 222 alanine to valine (677 C to T) and codon 429 glutamate to alanine (1298 A to C) in *MTHFR*; codon 22 isoleucine to methionine (66 A to G) in *MTRR*; and codon 487 glutamate (**1*) to lysine (**2*) in *ALDH2*.

Statistical Analysis

We excluded three non-adenocarcinoma cases (mucinous adenocarcinoma, squamous cell carcinoma, and final diagnosis as adenoma) and six matched controls. We then limited the study subjects to sufficient-blood sample donors (14 subjects were excluded) and those whose genotyping data were available. We failed to determine the genotypes of 12 samples because of an inadequate volume of abstracted DNA. This left 107 adenocarcinoma cases and 224 matched controls of analysis.

Characteristics of cases and controls were compared and tested by the Mantel-Haenszel test using matched-pair strata. We estimated odds ratios (ORs) and 95% confidence intervals (CIs) of colorectal cancer for folate, vitamin B_6, vitamin B_{12}, related-enzymatic SNPs, and the joint effect between nutrients and genotypes with the conditional logistic regression model on the matched case-control pairs. Nutrient intake was adjusted for total energy intake with the residual model (42) and divided into tertile categories based on the control subjects. In addition to controlling for sex, age, and hospitals in the statistical model, covariates for the adjustment of OR were smoking (never, 1–30 pack-years, 30 pack-years or more), alcohol consumption (never, past, current), body mass index (kg/m^2; tertiles based on controls), and total dietary fiber intake (tertiles based on controls, energy-adjusted using the residual method). We selected these confounding factors after checking whether they changed the ORs of the studied vitamins when entered in the statistical model (43). Other factors such as family history of colorectal cancer and red meat intake were not included in the final statistical model because these factors did not change the association of the studied vitamins with colorectal cancer when entered in the statistical model. To investigate whether related genotypes modified the effect of folate, vitamin B_6, vitamin B_{12}, or vitamin B_2, we mainly divided the subjects into individuals with variant (or mutant) alleles and those with two wild alleles and assessed the different effects of these nutrients by the genotype. *MTHFR* C677T genotypes were divided into homozygous mutant and others as same division as in most of the previous studies (13,14). Each different effect by the genotypes was tested with the log-likelihood ratio test using interaction terms between folate, vitamin B_6, vitamin B_{12}, or vitamin B_2 and genotypes (44). *P* values for all statistical tests were evaluated using the two-sided test with 0.05 as the significance level. All statistical analyses were conducted using the SAS program (SAS Institute, Cary, NC) (45).

Results

Folate and vitamin B_{12} intake were not statistically different between cases and controls (Table 1). Vitamin B_6 intake was higher in controls than in cases. Control subjects took in

Table 1. Characteristics of Cases and Controls[a]

	Unit	Case	Control	P^b
Number		107	224	
Sex (matching factor), men (%)		66 (62)	141 (63)	
Age (matching factor), mean (SD)	years	60 (9)	60 (9)	
Smoking, current (%)		25 (23)	43 (19)	0.41
Alcohol consumption, current (%)		58 (54)	151 (67)	0.013
Alcohol consumption, mean (SD)	g/day	19.8 (29.6)	17.7 (28.2)	0.57
BMI, mean (SD)	kg/m^2	22.8 (3.0)	23.7 (2.9)	0.032
Folate, mean (SD)	μg/day	417 (185)	439 (166)	0.14
Vitamin B_6, mean (SD)	mg/day	1.5 (0.3)	1.6 (0.3)	0.037
Vitamin B_{12}, mean (SD)	μg/day	9.3 (3.9)	9.9 (4.8)	0.20
Vitamin B_2, mean (SD)	mg/day	1.7 (0.5)	1.7 (0.5)	0.48
Total dietary fiber, mean (SD)	g/day	13.8 (6.5)	14.9 (6.3)	0.047
Red meat intake, mean (SD)	g/day	44.4 (32.4)	41.7 (23.5)	0.62
Total energy intake, mean (SD)	kcal/day	2,156 (735)	2,127 (755)	0.93
Family history of colorectal cancer (%)		15 (14)	20 (9)	0.22
Vitamin supplement use (%)		15 (14)	35 (16)	0.98

a: Abbreviations are as follows: SD, standard deviation; BMI, body mass index.
b: *P* for Mantel-Haenszel test with matched-pair strata.

more dietary fiber than case subjects. Body mass indices and percentage of current drinkers in cases were lower than in controls.

There were nonsignificantly inverse associations of *MTHFR* 677TT and 1298CC and *MTRR* 66GG with colorectal cancer (Table 2). *ALDH2* polymorphism showed a slight but not significantly increased risk of colorectal cancer.

Multivariate OR for folate, vitamin B_6, vitamin B_{12}, or vitamin B_2 was not statistically significant (Table 3). ORs for folate intake were 0.61 (95% CI = 0.32–1.1) for the second tertile and 0.55 (95% CI = 0.25–1.2) for the highest tertile (P for trend = 0.11) before being adjusted for total dietary fiber intake. After adjustment for dietary fiber intake, OR for the highest folate intake nonsignificantly increased (OR = 1.3; 95% CI = 0.49–3.4).

Statistical interaction (P for interaction = 0.04) was detected between folate intake and the *MTRR* polymorphism (Table 4). However, no linear trend was obtained in each polymorphism stratum. The association of folate with colorectal cancer did not differ by any genetic polymorphism of *MTHFR*.

Vitamin B_6 was associated with a decreased risk of colorectal cancer only in wild-type *MTRR* (Table 4). In heterozygous and homozygous mutant types of *MTRR*, a decreased risk was not observed for the higher intake of vitamin B_6 (P for interaction = 0.02). The highest tertile of vitamin B_6 intake was associated with a nonsignificant decreased risk only in the *MTHFR* 677TT genotype (OR = 0.39; 95% CI = 0.078–1.9), although the interaction was not statistically significant (P for interaction = 0.18). Two *MTHFR* polymorphisms did not statistically interact with vitamin B_6 intake in relation to colorectal cancer. Further, we tested the statistical interaction among folate intake, vitamin B_6 intake, and *MTRR* genotype. The interaction among these three variables was not significant (P for interaction = 0.48). The interaction between folate and the *MTRR* genotype was more important (P for interaction = 0.21) than that between vitamin B_6 and the *MTRR* genotype (P for interaction = 0.91) in this statistical model.

Table 2. Odds Ratios and 95% Confidence Intervals of Colorectal Cancer for Genotypes of Related Enzymes[a]

Gene	wt/wt	wt/mt	mt/mt	Trend P	mt[b]
MTHFR C677T	1.0 (reference)	0.75 (0.44–1.3)	0.79 (0.41–1.5)	0.44	0.95 (0.55–1.6)[c]
	32/51[d]	49/114	25/57		
MTHFR A1298C	1.0 (reference)	1.0 (0.58–1.7)	0.35 (0.040–3.0)	0.59	0.92 (0.54–1.6)
	73/156	32/63	1/5		
MTRR	1.0 (reference)	1.2 (0.72–1.9)	0.79 (0.27–2.3)	0.88	1.0 (0.70–1.5)
	58/128	44/82	5/14		
ALDH2	1.0 (reference)	1.1 (0.66–1.9)	1.2 (0.49–2.9)	0.60	1.1 (0.69–1.9)
	61/137	36/72	9/15		

a: Odds ratios on matched pair by matching factors. Abbreviations are as follows: wt, wild type; mt, mutant type.
b: wt/mt and mt/mt versus wt/wt.
c: mt/mt versus wt/wt and wt/mt.
d: Number of cases/number of controls.

Table 3. Odds Ratios[a] and 95% Confidence Intervals of Colorectal Cancer for Folate, Vitamin B_6, Vitamin B_{12}, and Vitamin B_2 Intake

Nutrient	Tertile 1	Tertile 2	Tertile 3	Trend P
Folate, mean (μg/day)	<343	343–484	485+	
	1.0 (reference)	1.0 (0.49–2.1)	1.3 (0.49–3.4)	0.62
	44/71[b]	32/78	31/75	
Vitamin B_6, mean (mg/day)	<1.46	1.46–1.74	1.75+	
	1.0 (reference)	1.1 (0.57–2.1)	0.88 (0.41–1.9)	0.77
	47/74	36/76	24/74	
Vitamin B_{12}, mean (μg/day)	<7.3	7.3–11.1	11.2+	
	1.0 (reference)	0.91 (0.48–1.7)	1.1 (0.55–2.2)	0.77
	36/72	37/77	34/75	
Vitamin B_2, mean (mg/day)	<1.49	1.49–1.84	1.85+	
	1.0 (reference)	0.87 (0.42–1.8)	1.1 (0.52–2.5)	0.64
	44/74	27/73	36/77	

a: Odds ratios on matched pairs by matching factors and adjusted for smoking (never, <30 pack-years, 30 pack-years or more), alcohol consumption (never, past, current), body mass index (tertiles based on controls), and total dietary fiber intake (tertiles based on controls).
b: Number of cases/number of controls.

Table 4. Odds Ratios[a] and 95% Confidence Intervals of Colorectal Cancer for the Combination Between Folate, Vitamin B_6, Vitamin B_{12}, or Vitamin B_2 Intake and Genotypes of Related Enzymes

Gene		Tertile 1	Tertile 2	Tertile 3	P^b
Folate					
MTHFR C677T	CC+CT	1.0 (reference)	1.1 (0.46–2.4)	1.4 (0.48–3.9)	
		33/53[c]	23/57	25/55	
	TT	1.2 (0.42–3.2)	1.2 (0.39–3.7)	1.2 (0.30–4.5)	0.91
		10/17	9/20	6/20	
MTHFR A1298C	AA	1.0 (reference)	0.82 (0.34–2.0)	1.1 (0.37–3.4)	
		30/48	21/54	22/54	
	AC+CC	0.83 (0.31–2.2)	1.3 (0.44–3.8)	0.93 (0.26–3.4)	0.63
		14/23	11/24	8/21	
MTRR A66G	AA	1.0 (reference)	1.8 (0.70–4.6)	1.1 (0.36–3.5)	
		25/46	20/38	13/44	
	AG+GG	1.3 (0.56–3.1)	0.71 (0.27–1.8)	2.1 (0.71–6.5)	0.043
		19/25	12/40	18/31	
Vitamin B_6					
MTHFR C677T	CC+CT	1.0 (reference)	1.1 (0.51–2.3)	1.2 (0.50–2.7)	
		32/57	27/56	22/52	
	TT	1.7 (0.62–4.6)	1.2 (0.45–3.5)	0.39 (0.078–1.9)	0.18
		14/16	9/20	2/21	
MTHFR A1298C	AA	1.0 (reference)	1.4 (0.64–3.2)	1.1 (0.43–2.8)	
		32/54	26/52	15/50	
	AC+CC	1.6 (0.61–4.1)	0.96 (0.35–2.7)	1.2 (0.39–3.6)	0.45
		15/20	9/24	9/24	
MTRR A66G	AA	1.0 (reference)	0.49 (0.20–1.2)	0.59 (0.23–1.5)	
		30/37	14/48	14/43	
	AG+GG	0.44 (0.19–1.02)	1.1 (0.51–2.6)	0.62 (0.22–1.7)	0.021
		17/37	22/28	10/31	
Vitamin B_{12}					
MTHFR C677T	CC+CT	1.0 (reference)	1.1 (0.51–2.3)	1.4 (0.62–3.2)	
		22/53	31/57	28/55	
	TT	1.7 (0.64–4.5)	0.81 (0.25–2.6)	1.0 (0.32–3.3)	0.43
		13/18	6/20	6/19	
MTHFR A1298C	AA	1.0 (reference)	0.81 (0.38–1.8)	0.99 (0.41–2.4)	
		27/48	26/60	20/48	
	AC+CC	0.66 (0.23–1.9)	1.1 (0.38–3.1)	1.1 (0.40–2.9)	0.60
		8/24	11/17	14/27	
MTRR A66G	AA	1.0 (reference)	1.4 (0.55–3.5)	1.3 (0.49–3.2)	
		16/42	23/42	19/44	
	AG+GG	1.5 (0.60–3.7)	0.88 (0.35–2.2)	1.6 (0.59–4.5)	0.40
		20/30	14/35	15/31	
Vitamin B_2					
MTHFR C677T	CC+CT	1.0 (reference)	1.0 (0.45–2.4)	1.3 (0.56–3.1)	
		31/54	22/51	28/60	
	TT	1.4 (0.51–3.8)	0.77 (0.23–2.5)	1.3 (0.39–4.3)	0.74
		12/19	5/22	8/16	
MTHFR A1298C	AA	1.0 (reference)	0.59 (0.24–1.4)	1.2 (0.49–3.0)	
		33/54	14/52	26/50	
	AC+CC	0.56 (0.19–1.6)	1.5 (0.55–3.8)	0.91 (0.32–2.6)	0.093
		10/20	13/21	10/27	
MTRR A66G	AA	1.0 (reference)	0.90 (0.36–2.2)	1.2 (0.48–2.9)	
		24/43	13/41	21/44	
	AG+GG	1.1 (0.49–2.5)	0.91 (0.35–2.3)	1.2 (0.45–3.3)	0.99
		20/31	14/32	15/33	

a: Odds ratios on matched pairs by matching factors and adjusted for smoking (never, <30 pack-years, 30 pack-years or more), alcohol consumption (never, past, current), body mass index (tertiles based on controls), and total dietary fiber intake (tertiles based on controls).

b: *P* for interaction.

c: Number of cases/number of controls.

Table 5. Odds Ratios[a] and 95% Confidence Intervals of Colorectal Cancer for the Combination Between Folate and Alcohol Consumption and Genetic Polymorphisms of Aldehyde Dehydrogenase

		Folate Intake			
		Tertile 1	Tertile 2	Tertile 3	P^b
Alcohol consumption	Never	1.0 (reference)	0.64 (0.13–3.2)	0.95 (0.21–4.4)	
		8/6[c]	10/22	21/36	
	Past + current	0.40 (0.12–1.4)	0.39 (0.11–1.4)	0.39 (0.084–1.8)	0.83
		26/65	22/56	10/39	
ALDH2	**1*1*[d]	1.0 (reference)	1.3 (0.54–3.1)	1.7 (0.53–5.2)	
		23/49	21/45	17/43	
	**1*2 +*2*2*[d]	1.4 (0.52–3.7)	0.93 (0.31–2.8)	1.2 (0.37–3.8)	0.50
		21/22	11/33	13/32	

a: Odds ratios on matched pairs by matching factors and adjusted for smoking (never, <30 pack-years, 30 pack-years or more), body mass index (tertiles based on controls), and total dietary fiber intake (tertiles based on controls).
b: *P* for interaction.
c: Number of cases/number of controls.
d: **1*, wild-type glutamate allele; **2*, mutant-type lysine allele.

No statistical interaction was observed in the combination between vitamin B_{12} or vitamin B_2 intake and any genotype of the related enzymes (Table 4).

There was no interaction between folate intake and alcohol consumption or the *ALDH2* genetic polymorphism (Table 5).

Discussion

We investigated the association of folate, vitamin B_6, vitamin B_{12}, and vitamin B_2 and genotypes of related enzymes with colorectal cancer. Our results suggested that the genetic polymorphism of *MTRR* may interact with folate and vitamin B_6 in relation to colorectal cancer.

Le Marchand et al. (16) reported that the *MTRR* A66G polymorphism was associated with colorectal cancer and did not interact with any nutrient, including folate, vitamin B_6, vitamin B_{12}, or vitamin B_2. The result of the present study suggested that higher vitamin B_6 intake may be useful only for wild-type *MTRR*. Although the folate and *MTRR* polymorphism interaction with colorectal cancer incidence was statistically significant, the effect of the interaction was difficult to interpret. The function of this polymorphism of *MTRR* is still unknown, and evidence on this polymorphism needs to be accumulated in colorectal cancer epidemiology as well as the experimental study area on carcinogenesis and prevention.

Our study did not suggest an inverse association of folate intake in the *MTHFR* variant genotype rather than the 677CC genotype. In fact, most previous studies failed to detect any statistical interaction between folate intake and the *MTHFR* C677T polymorphism (16–19,46–49). Previous studies revealed that the TT genotype with higher plasma folate was strongly inversely associated with risk (13,14). Both (13,14) were derived from the Physician's Health Study, which reported that age-adjusted OR for the folate rich and TT genotype was 0.32 (95% CI = 0.15–0.68) (13). In a different report, the age-adjusted OR for folate rich in TT genotype was 0.29 (95% CI = 0.12–0.73) compared with the CC/CT genotype (14). In addition, the inverse association of folate intake was not necessarily consistent among all studies (25,26, 50–53), possibly because other preventive factors may confound the association of folate intake with colorectal cancer. In fact, dietary fiber intake strongly confounded the association of folate intake with colorectal cancer in our study. Pearson's correlation coefficient was 0.83 between energy-adjusted folate and dietary fiber intake. This may be because the main food sources were the same between folate and dietary fiber, such as rice, cabbage, spinach, and Japanese white radish ("Daikon") (54). Folate appeared to be associated with colorectal cancer before being adjusted for dietary fiber intake. After the adjustment for fiber intake, however, the association of folate disappeared. That part of the evidence from some previous studies that showed the inverse association of folate intake may be due to a confounding with dietary fiber because not all studies considered fiber intake (25,52). Another possible reason is that folate intake may not reflect the absorbed and functional folate in the body. Folate absorption may be modified by alcohol consumption (21,22) and bowel microflora (30). Although folate intake calculated by the questionnaire correlated highly with folate intake estimated from DRs, plasma folate did not correlate highly with these records (rank correlation = 0.19). Therefore, the plasma folate concentration, which reflects the available folate, may more clearly demonstrate an interaction with a related genetic polymorphism rather than folate intake itself.

Many studies reported that folate deficiency was associated with the higher risk of colorectal cancer in drinkers than in nondrinkers (23–26). Our study did not confirm this evi-

dence. Moreover, our results showed that alcohol consumption decreased the risk of colorectal cancer, although not significantly. This was inconsistent with previous studies (13, 14,23,24). One possible reason was that some past drinkers were misclassified as never-drinkers. High-risk individuals may be included in the low-risk group, and the association appeared to be the converse direction of an alcohol-risk hypothesis. To our knowledge, our study is the first to evaluate the interaction between folate intake and the *ALDH2* polymorphism. The *ALDH2* genetic polymorphism exerts low enzyme activity (28) and causes high blood levels of acetaldehyde (29). Acetaldehyde possibly induces cleavage of folate (22,30) and inhibits its supply. Therefore, we hypothesized that subjects with the *ALDH2* mutant allele had a higher probability of folate deficiency than subjects without the mutant allele because acetaldehyde, which induces folate cleavage, may tend to stay in the blood circulation of those with the mutant allele after drinking. We expected that low folate with the *ALDH2* mutant allele would be associated with colorectal cancer more than that without the mutant allele. However, no significant interaction was obtained. Because the *ALDH2* genotype was associated with alcohol-drinking behavior (55), further larger studies should stratify study subjects by alcohol consumption to examine the interaction of the *ALDH2* genotype in the association between folate intake and colorectal cancer.

If study subjects had any symptoms, we asked both cases and controls to reply to us their existing dietary habits prior to their present symptoms. Because there were probably more study cases with present symptoms than controls, cases may have had to recall a more distant past than controls. Disease experience may also affect recall, and cases may more earnestly seek to recall dietary habits than controls. Recalling the more distant past or recalling it more earnestly in cases may lead to differential misclassification. Moreover, information on the distant past in cases may be more vague than in controls, leading to underestimation of the effects of nutrients. On the other hand, the more accurate information on dietary habits by the more earnest recall of cases than controls may result in overestimation of the effects of nutrients. We did not assess symptom-related changes in dietary habit. Our small sample size may limit the interpretation of our results. We may have failed to demonstrate the association and the evaluation of gene–nutrient interactions. Because almost all subjects participated in this study, nonresponse bias may be small. However, controls were selected from medical checkup examinees who were interested in their health and screening. Therefore, there may be a self-selection bias by the healthy volunteer effect. We intended to investigate another genetic polymorphism, methionine synthase A2756G. This enzyme is a member of the folate metabolic pathway, and this genetic polymorphism may modify the effect of folate or related nutrients on colorectal cancer (13,16). Although we performed *MTR* genotyping, we failed to obtain reliable data on this polymorphism.

In conclusion, the present study did not support the existing hypothesis of gene–nutrient interaction and inverse association of folate intake with colorectal cancer nor did it show any significant association of vitamin B_6, vitamin B_{12}, vitamin B_2, or related-enzyme genetic polymorphisms.

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2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) level in human hair as biomarkers for dietary grilled/stir-fried meat and fish intake

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Abstract

Several case-control studies have reported possible associations between heterocyclic amine (HCA) intake and the risk of cancer. However, the validity of a questionnaire to assess HCA intake has hardly been examined. In particular, no biomarker which could serve as an independent measure of habitual HCA intake has been established. Therefore, the validity of a questionnaire to assess HCA intake by means of a biomarker remains to be investigated. In this study, we examined the availability of hair HCAs as a biochemical indicator of dietary intake of HCAs. Study subjects were 20 volunteers (7 men and 13 women) aged 25–57 years, either residents of Tokyo or the neighboring cities in Japan. We collected individual weighed dietary records (DR) over 28 consecutive days. Approximately 3–5 g of hair was collected twice from all subjects before and after DR at intervals of 1–3 months. The mean (S.D.) 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) level of hair was 1376.0 pg/g hair (928.9) and 16.6 ng/g melanin (12.3). A steady increase in the mean PhIP level in hair from the lowest to the highest tertile of the grilled/stir-fried meat intake was observed ($P=0.009$), but not in the grilled/stir-fried fish intake ($P=0.461$). The PhIP level in hair was highly correlated with the grilled/stir-fried meat intake ($r=0.68$) but not with the grilled/stir-fried fish intake ($r=0.28$). These observations were made of hair with and without melanin adjustment. The present study indicates that the PhIP level in hair can be used as a biological indicator of dietary intake of HCAs.

Keywords: Heterocyclic amines; Biomarker; Dietary record; Human hair

1. Introduction

Although some heterocyclic amines (HCAs) are known to be genotoxic in mammalian cell lines [1,2] and carcinogenic in experimental animals [3–5], the epidemiologic evidence for an etiologic role of HCAs in carcinogenesis is inconsistent [6–10]. One of the prime reasons for this inconsistency is the difficulty of assessing human exposure to HCAs. Because the concentrations of HCAs depend on the cooking method or doneness level of meat or fish, the development of a complete and standardized database of the concentrations of HCAs is difficult, and the estimation of dietary

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HCAs from a questionnaire is likely to be misclassified. Although a database for HCA content has recently been developed for 297 food items [11], these limited food items may present difficulties in estimating the dietary HCAs for different study populations. Thus, measuring HCAs in biological samples offers an attractive alternative approach to estimating human exposure to these substances. Several studies have used human urine levels as an indicator of HCA intake [12,13]. However, urinary metabolites will not provide an accurate assessment of usual HCA intake, because the half-life of HCA metabolite is not enough to accurately assess them in a regular diet [14,15]. Although HCAs in hair samples are reportedly available to determine the HCA contents in regular diet [16], they have been so far compared only with the frequency of meat intake and not quantitative dietary intake.

We have previously reported the ability of an analytical method to detect the 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) level in human hair [17]. It has long been known that PhIP interacts with melanin [18]. Besides, there is a report indicating the role of eumelanin for PhIP uptake into hair, and the PhIP content in the hair based on melanin content tends to negate the effect of melanin content variation on the concentration of PhIP in the hair [14]. Further, we reported a positive correlation between PhIP levels in hair and melanin content in hair in the previous study [17]. Then, in the present study, we examined the availability of PhIP in hair as an indicator of dietary intake of HCAs. The PhIP levels in hair or PhIP levels per melanin content were compared with grilled/stir-fried meat or fish intake from dietary records over 28 days.

2. Materials and methods

2.1. Study population

Study subjects were 20 healthy volunteers (7 men and 13 women) aged 25–57 years, non-smokers, and residents of Tokyo or the neighboring cities in Japan. All subjects gave their informed consent to participate in this study. Mean (S.D.) age was 38.1 (8.6) years and mean (S.D.) body mass index was 21.8 (2.6). We collected individual weighed dietary records (DR) over 28 consecutive days. Approximately 3–5 g of hair was collected twice from all subjects before and after DR at intervals of 1–3 months. Ten (50%) subjects had dyed hair.

2.2. Analysis of hair HCAs

Details of the analysis have been reported elsewhere [17]. Briefly, 3 g of hair was washed in 0.1% SDS (100 ml) by ultrasonication for 5 min, after which the liquid was decanted, and hair was then washed four times with water (100 ml), once with 100% ethanol (50 ml), and dried at room temperature [19]. The dried hair sample was weighed, and 1N NaOH (100 ml) and 10 ng of internal standard (PhIP-d_3) were added. This solution was incubated at 100 °C for 45 min in a capped container (225 ml graduated conical tube with polypropylene cup, Falcon, NJ, USA), and the tube was capped loosely. After centrifugation at 3100 rpm for 10 min, the supernatant was filtrated (5B filter, ADVAN-TEC, Tokyo, Japan), and then neutralized to pH 7–9 with 6N HCl. PhIP in this filtrate was extracted using a Blue-Chitin column absorption method [20]. Briefly, HCAs absorbed on a Blue-Chitin column were eluted with 20 ml of MeOH–28% NH_3 (50:1) (flow rate, 5 ml/min). The eluate was concentrated under vacuum at room temperature using a centrifugal concentrator CC-105 with low temperature trap TV-105 (TOMY, Tokyo, Japan). The residue was then dissolved in 1 ml of 100% MeOH. After centrifugation of 3100 rpm for 10 min, the supernatant was collected, and then concentrated under vacuum. The residue was dissolved in 2 ml of 0.1N HCl. After washing with 2 ml of *n*-hexane, the aqueous layer was adjusted to pH > 10 with 28% NH_3, and then extracted twice with 2 ml of dichloromethane. The organic layer was concentrated under vacuum. The residue was dissolved in 500 μl of 40 μM ammonium acetate: 100% MeOH (1:1, v/v), and then filtrated with 0.45 μM filter (Ultrafree-MC, Millipore, Bedford, MA, USA).

The column-switching liquid chromatography–mass spectrometry (LC–MS) system was LCMS-2010A coupled with a column switching system (Co-sense for BA system, Shimadzu, Kyoto, Japan). A SIL-10APvp automatic injector equipped with a contamination control kit (Shimadzu, Kyoto, Japan) was used. Three hundred microliters of the sample was injected by an auto-sampler, and was loaded onto the extraction column (Shim-pack MAYI-ODS column, 2.0 mm × 10 mm, Shimadzu, Kyoto, Japan) by 10 mM ammonium acetate at a rate of 2.0 ml/min for 4 min. Then, the valve was switched. The analyte was introduced into the analytical column (Mercury MS LUNA 3 μm C18 column, 2.0 mm × 20 mm, Penomenex, Torrance, CA) at a rate of 0.2 ml/min. Then 40 μM ammonium acetate (pH 4.0, A) and methanol (B) were used as an analytical mobile phase. The gradient program was as follows: 13% B (0–5 min)–45% B (5.01 min)–45% B (5.01–13 min)–13% B (13.01 min). The column oven was maintained at 40 °C. The mass spectrometry conditions for electrospray ionization (ESI)-MS were as follows: drying nitrogen gas temperature was set at 250 °C and introduced into the capillary at a flow rate of 4.5 l/min; the capillary was held at a potential of 4.5 kV for the positive ion mode. In selected ion monitoring (SIM) mode, ions at m/z 225 were assigned to $[M+H]^+$ of PhIP.

Hair sample (1 mg) and sepia melanin (1 mg) were dissolved in 1 ml of a mixture of Soluene-350 and water (9:1, v/v), followed by heating at 95 °C for 45 min. Optical density was observed at 500 and 650 nm (A_{500} and A_{650}) in Jasco V-550 (Japan Spectroscopic, Tokyo, Japan). A_{500} indicates the quantity of total melanin, and the ratio A_{650}/A_{500} equals the ratio of eumelanin to total melanin in the hair sample. Total melanin

concentrations of hair samples were calculated according to a previous method [21].

2.3. Meat and fish intakes assessed by DR

Weighed DR were collected over 28 consecutive days. The subjects were asked to provide detailed descriptions of meat (beef, pork, chicken, and processed meat) and fish (any fish in fresh, dried, and processed) including the method of preparation (grilled, stir-fried, deep fried, boiled, fresh, and others) and intake of burnt portion. The subjects reported doneness levels (charred, well-done, medium well-done, medium, medium rare, rare) for grilled fish or meat. Grilled or stir-fried fish and meat intake were calculated as a surrogate indicator for HCA intake, because the database of HCA content is not sufficient to estimate HCA intake. We did not include processed fish in total fish, because the burnt portion of grilled or stir-fried processed fish which was made from minced flesh, egg, starch, and other seasonings was not the burnt portion of fish.

2.4. Data analysis

The statistical analyses were performed using SAS (version 9.1; SAS Institute, Inc., Cary, NC). The mean PhIP level in hair which was collected twice was computed and expressed as the crude level (pg/g hair) and level per melanin content (ng/g melanin). To assess whether PhIP in hair can serve as a valid biomarker in epidemiological studies, subjects were categorized into three groups according to tertiles of grilled and stir-fried fish and meat intake from DR. Mean PhIP in hair in different tertile of DR value was computed. Tests for trends of PhIP in hair were conducted using the mantel extension test. Spearman rank correlations were used to assess the degree of association between PhIP in hair and grilled or stir-fried fish and meat intake from DR. Association between PhIP in hair and intake of the burnt portion of fish, and the frequency of the preferable doneness level when the meat was grilled or stir-fried were also examined.

3. Results

The mean (S.D.) PhIP level in hair was 1376.0 pg/g hair (928.9) and 16.6 ng/g melanin (12.3).

Table 1 shows meat and fish intake of grilled/stir-fried meat and fish intake assessed with DR of the study population. Daily meat intake was 86.7 g and daily grilled/stir-fried meat intake was 47.7 g. Grilled/stir-fried beef and pork intake was almost the same or greater than those of grilled/stir-fried chicken and processed meat. Daily fish intake was 48.4 g and daily grilled/stir-fried fish intake was 21.8 g. Grilled/stir-fried and dried fish intake was far less than grilled/stir-fried raw fish intake. Frequency of burnt portion in fish intake was 6.2 (times/month) and well-done meat intake was 7.5 (times/month).

Table 2 shows the mean PhIP level in hair according to the tertiles of the grilled/stir-fried meat or fish intake assessed with DR. A steady increase was observed in the mean PhIP level in hair from the lowest to the highest tertile of the pork ($P=0.035$), chicken ($P=0.020$), total meat ($P=0.009$), and total meat and fish intake ($P=0.004$), but not for grilled/stir-fried fish intake ($P=0.461$). These observations were found with or without hair adjustment with melanin. Table 2 also presents the correlation between the PhIP level in hair and grilled/stir-fried meat and/or fish intake assessed

Table 1
Meat and fish intake assessed with DR[a] of the study population ($n=20$)

	Mean ± S.D.	Percentile		
		25th	50th	75th
Meat and fish intake assessed with DR				
Total meat (g/day)	86.7 ± 40.1	53.6	69.1	113.6
Grilled/stir-fried meat (g/day)				
Total meat	47.7 ± 20.3	33.3	46.0	56.2
Beef	16.9 ± 11.2	10.2	15.2	18.7
Pork	17.7 ± 9.3	11.7	17.9	22.4
Chicken	9.7 ± 8.3	2.9	7.0	14.1
Processed meat	3.4 ± 3.0	0.8	2.5	5.4
Well-done meat (times/month)	7.5 ± 8.9	1.0	5.0	9.0
Total fish (g/day)	48.4 ± 23.2	31.6	40.5	60.3
Grilled/stir-fried fish (g/day)				
Total fish	21.8 ± 12.3	11.2	20.1	28.2
Raw fish	20.1 ± 11.4	11.2	17.5	26.5
Dried fish	1.7 ± 2.7	0.0	0.0	2.7
Burned portion of fish (times/month)	6.2 ± 4.7	2.5	4.5	11.0

[a] Dietary records.

Table 2
PhIP[a] level in hair according to tertiles of grilled/stir-fried meat and fish intake assessed with DR[b] and their correlation ($n = 20$)

	Crude levels (pg/g hair)			Levels per melanin content (ng/g melanin)		
	Mean (95% CI)	Spearman correlation		Mean (95% CI)	Spearman correlation	
		r	P		r	P
Beef		0.34	0.137		0.48	0.031
Lowest	830.7 (53.5, 1607.9)			8.6 (0, 18.4)		
Second	1626.9 (907.4, 2346.4)			16.8 (7.7, 25.9)		
Highest	1592.3 (872.8, 2311.9)			23.1 (14.1, 32.2)		
P for trend[c]	0.152			0.034		
Pork		0.46	0.039		0.39	0.091
Lowest	891.1 (158.0, 1624.2)			10.7 (0, 21.3)		
Second	1200.0 (521.2, 1878.7)			19.0 (9.1, 28.9)		
Highest	1967.5 (1288.8, 2646.3)			19.1 (9.3, 29.0)		
P for trend[c]	0.035			0.229		
Chicken		0.53	0.017		0.52	0.019
Lowest	960.8 (286.6, 1634.9)			11.6 (3.1, 20.0)		
Second	1062.8 (479.0, 1646.6)			11.2 (3.9, 18.5)		
Highest	2208.7 (1534.6, 2882.8)			28.7 (20.2, 37.1)		
P for trend[c]	0.020			0.017		
Processed meat		0.15	0.536		0.13	0.580
Lowest	1694.7 (884.5, 2504.9)			17.7 (7.0, 28.3)		
Second	1053.9 (303.8, 1804.0)			11.6 (1.7, 21.4)		
Highest	1424.8 (674.7, 2174.8)			20.6 (10.7, 30.4)		
P for trend[c]	0.638			0.629		
Total meat		0.68	0.001		0.69	0.001
Lowest	721.7 (44.7, 1398.6)			8.9 (0.2, 17.7)		
Second	1257.9 (631.2, 1884.7)			13.1 (5.0, 21.2)		
Highest	2054.8 (1428.1, 2681.5)			26.6 (18.5, 34.7)		
P for trend[c]	0.009			0.009		
Raw fish		0.20	0.391		0.18	0.448
Lowest	1355.3 (510.1, 2200.4)			13.7 (2.7, 24.6)		
Second	1345.8 (563.3, 2128.2)			15.4 (5.2, 25.5)		
Highest	1423.8 (641.4, 2206.3)			20.2 (10.1, 30.3)		
P for trend[c]	0.891			0.333		
Dried fish		0.09	0.709		−0.03	0.893
Lowest	1428.7 (840.6, 2016.8)			19.2 (11.3, 27.1)		
Second	476.0 (0, 1855.2)			7.5 (0, 26.1)		
Highest	1550.1 (812.9, 2287.4)			14.9 (5.0, 24.8)		
P for trend[c]	0.868			0.425		
Total fish		0.28	0.230		0.23	0.330
Lowest	1355.3 (548.6, 2161.9)			13.7 (3.3, 24.1)		
Second	1059.7 (312.9, 1806.5)			12.8 (3.2, 22.4)		
Highest	1709.9 (963.1, 2456.7)			22.8 (13.2, 32.4)		
P for trend[c]	0.461			0.170		
Total meat and fish		0.66	0.001		0.65	0.002
Lowest	615.1 (0, 1254.2)			8.1 (0, 17.0)		
Second	1317.3 (725.6, 1909.0)			14.4 (6.1, 22.6)		
Highest	2086.7 (1495.1, 2678.4)			26.0 (17.8, 34.2)		
P for trend[c]	0.004			0.008		

[a] 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.
[b] Dietary records.
[c] Mantel extension test for difference.

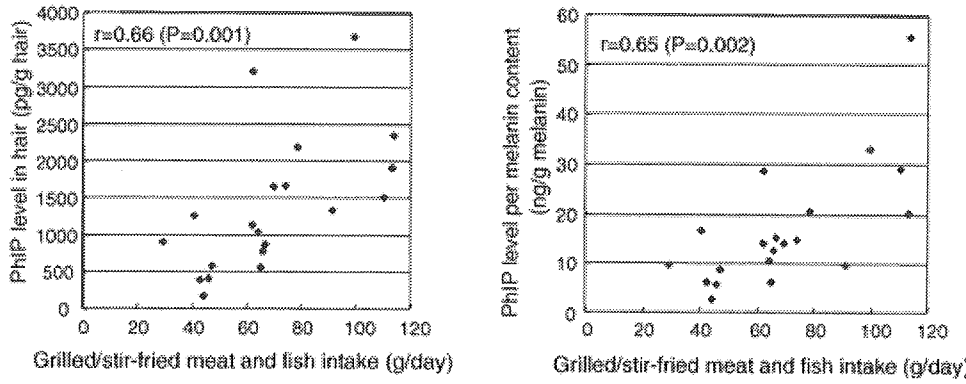

Fig. 1. Scatter plot of PhIP level in hair (crude levels or levels per melanin content) vs. dietary grilled/stir-fried meat and fish intake ($n = 20$).

with DR. As seen in Fig. 1, the PhIP level in hair was highly correlated with intakes of the amount of the grilled/stir-fried meat and fish together ($r = 0.66$). This observation also applies to the PhIP level per melanin content ($r = 0.65$). The PhIP level in hair and the PhIP level per melanin content were also highly correlated with the amount of grilled/stir-fried total meat intake ($r = 0.68$ and 0.69, respectively) but was not correlated with the amount of grilled/stir-fried fish intake ($r = 0.28$ and 0.23, respectively).

The PhIP level in hair and frequency of the burnt portion in fish intake were not correlated ($r = 0.02$). The PhIP level in hair and frequency of preferable doneness level when the meat was grilled or stir-fried were also not correlated ($r = 0.02$).

4. Discussion

The present study is the first to examine the availability of HCAs in hair as a biochemical indicator of dietary intake of HCAs using the grilled/stir-fried meat or fish intake from 28 days DR. Although the PhIP level in hair did not correspond to the amount of grilled/stir-fried fish intake by DR, it corresponded to the amount of grilled/stir-fried meat or grilled/stir-fried meat and fish combined.

In epidemiological studies, biochemical indicators of dietary HCA intake have great roles as an independent measure of HCA intake, which is independent of dietary assessment error and could account for the variability due to foods, cooking method or doneness level. In previous studies, human urine has been investigated as an indicator of HCA intake [12,13]. However, the half-life of the HCA metabolites is <12 h and reflects recent dietary intake [14,15]. The urinary metabolites will therefore not provide an accurate assessment of usual HCA intake. Hair has often been used for tests of drug abuse, because drugs in hair have a long half-life compared with those in urine or blood [22–24]. HCAs in hair samples are reportedly available to determine the HCA contents in regular diet [16]. However, in Reistad's report, HCAs in hair samples were compared with frequency of meat intake and not with quantitative dietary intake.

Foods which contribute to HCA intake differ with the generation or area in Japan. Because subjects in this study were relatively young and lived in the suburbs of Tokyo, they might have consumed more meat and less fish than the general population. Daily meat and fish intake in these subjects were thus compared with those of subjects in a large-scale population-based prospective study in Japan (JPHC study) [25,26]. For instance, daily meat intake was 86.7 g and daily grilled/stir-fried meat intake was 47.7 g in the present study, against daily meat intake of 58.3 g and daily grilled/stir-fried meat intake of 31.4 g in the JPHC study. Daily fish intake was 48.4 g and daily grilled/stir-fried fish intake was 21.8 g in the present study, against daily fish intake of 83.2 g and daily grilled/stir-fried fish intake of 54.7 g in the JPHC study. These low grilled/stir-fried fish intakes inevitably lead to the low HCA intake. Besides, it has been reported that HCA composition differs in terms of the type of fish, and that the HCA level in fish skin is higher than in fish flesh [26]. As possible reasons why the amount of grilled/stir-fried meat intake significantly correlated with the PhIP level in hair whereas the grilled/stir-fried fish intake did not, there was little high grilled/stir-fried meat intake and low grilled/stir-fried fish intake, and especially little dried fish intake in which the cooking method was almost always grilling. It was also possible that subjects were relatively health-conscious and tended not to eat grilled fish skin. However, an association between the PhIP level in hair and combined grilled/stir-fried meat and fish was observed in the present study. It is highly