One-carbon metabolism-related gene polymorphisms and risk of head and neck squamous cell carcinoma: Case–control study

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Low consumption of vegetables and fruits, which leads to insufficient folate intake, is associated with increased risk of several types of cancer, including head and neck squamous cell carcinoma (HNSCC). Functional polymorphisms in genes encoding one-carbon metabolism enzymes, such as methylenetetrahydrofolate reductase (*MTHFR* C677T and A1298C), methionine synthase (*MTR* A2756G), methionine synthase reductase (*MTRR* A66G) and thymidylate synthase (*TS*), influence folate metabolism and thus might impact on HNSCC risk. We conducted a case-control study with 237 HNSCC cases newly and histologically diagnosed and 711 age- and sex-matched non-cancer controls to clarify associations with these five polymorphisms. Gene–environment interactions between polymorphisms and smoking and drinking habit and folate consumption were also evaluated by logistic regression analysis. Dietary folate intake was inversely associated with HNSCC risk. None of the polymorphisms showed any significant impact on HNSCC risk by genotype alone, but we found interactions between drinking habit and *MTHFR* C667T ($P = 0.04$), *MTR* A2756G ($P = 0.04$) and *MTRR* A66G ($P = 0.03$) polymorphisms. The results suggest that there may be interactions between one-carbon metabolism-related polymorphisms and alcohol drinking for HNSCC risk. (*Cancer Sci* 2007; 98: 1439–1446)

Head and neck squamous cell carcinoma (HNSCC) is common in several regions of the world in which there is extensive use of tobacco and high consumption of alcohol.[1] Mortalities of oral and pharyngeal cancers in Japan have increased constantly from 1960 to 2000 and reached those of western countries in the late 1990s.[2] Therefore, efforts toward primary prevention in addition to early detection have come under the spotlight.

Low dietary intake of vegetables and fruits is recognized as a risk factor for HNSCC,[3–7] suggesting that folate, which is plentiful in vegetables and fruits, is a protective nutrient.[8–11] Biological functions of folate within so-called 'one-carbon metabolism' are to facilitate *de novo* deoxynucleoside triphosphate synthesis and to provide methyl groups required for intracellular methylation reactions. Folate deficiency is thought to increase the risk of cancer through impaired DNA repair synthesis and disruption of DNA methylation that may lead to protooncogene activation.[12–14]

Methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR) and thymidylate synthetase (TS) play important and interrelated roles in folate metabolism (Fig. 1). The MTHFR reduces 5,10-methylenetetrahydrofolate (5,10-methylene THF) to 5-methyl THF, the primary circulating form of folate.[15] The TS catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) using 5,10-methylene THF.[16] The MTHFR product, 5-methyl THF, is the methyl group donor for the remethylation of homocysteine to methionine catalyzed by MTR.[17] MTR activity is maintained by MTRR.[18]

Polymorphisms in the genes for *MTHFR* C677T and A1298C, *MTR* A2756G, *MTRR* A66G and *TS* 28-bp variable number of tandem repeat (VNTR) are known to have functional relevance.[19] Variant alleles of the *MTHFR* C677T and A1298C polymorphisms generate an enzyme with reduced activity compared with wild type.[20,21] *MTR* A2756G heterozygote and homozygous variant genotypes are associated with increased plasma folate levels, and *MTRR* A66G variant alleles generate an enzyme with lower affinity for MTR.[22,23] *TS* with triple repeat alleles is associated with greater TS expression compared with the double repeat.[24] Thus they might play roles in the etiology of HNSCC in combination with environmental factors. A previous case-control study in the USA reported that the *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G polymorphisms were associated with HNSCC risk,[25,26] whereas the *MTHFR* C677T and *TS* polymorphisms demonstrated no link.[25,27] Because information on HNSCC is limited regarding these polymorphisms,[25–28] we conducted the present case-control study, taking into consideration alcohol drinking, tobacco smoking and intake of folate.

Materials and Methods

Subjects. The cases were 237 patients who were newly and histologically diagnosed as having HNSCC between January 2001 and June 2005 at Aichi Cancer Center Hospital in Japan and who did not have any earlier history of cancer. Malignant neoplasms of the salivary glands, nasal and paranasal were excluded from the present study as they have quite distinct natural histories and poorly understood etiologies. Controls ($n = 711$) were selected randomly and matched by age (±3 years) and sex to cases with a 1:3 case-control ratio. All of the subjects were recruited in the framework of the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC), as described elsewhere.[29,30] In brief, information on lifestyle factors was collected using a self-administered questionnaire, checked by a trained interviewer. Outpatients were also asked to provide blood samples. Each patient was asked about his or her lifestyle when healthy or before the current symptoms developed. Approximately 95% of eligible subjects complete the questionnaire and 60% provided blood

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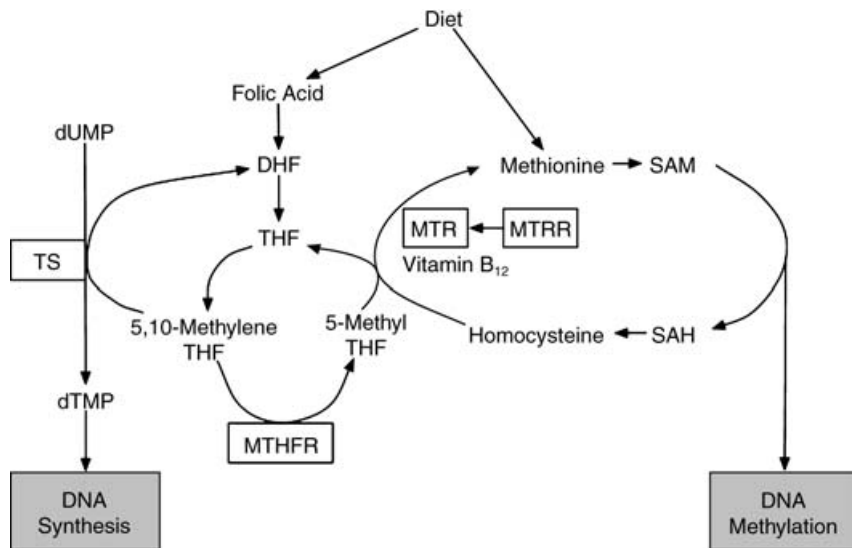[7]To whom correspondence should be addressed. E-mail: kmatsuo@aichi-cc.jp

Fig. 1. Overview of folate metabolism. Enzymes with polymorphisms investigated in the present study are boxed. DHF, dihydrofolate; dTMP, deoxythymidine monophoshate; dUMP, deoxyuridine monophoshate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

samples. Our previous study showed that the lifestyle patterns of first-visit outpatients accorded with those in a randomly selected sample of the general population of Nagoya City.[31] The data were loaded into a HERPACC database and linked routinely with the hospital-based cancer registry system to update the data on cancer incidence. All participants gave written informed consent and the study was approved by the Ethical Committee of Aichi Cancer Center.

Genotyping of MTHFR, MTR, MTRR and TS. DNA of each subject was extracted from the buffy coat fraction using BioRobot EZ1 and an EZ1 DNA Blood 350 mL Kit (Qiagen, Tokyo, Japan). The genotyping method was described in our previous reports with the polymerase chain reaction (PCR) TaqMan method using the GeneAmp PCR System 9700 or the 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Briefly, for the *MTHFR* C677T (dbSNP ID: rs677) and A1298C (rs1801131), as well as *MTR* A2756G (rs1805087) and *MTRR* A66G (rs1801394) polymorphisms, extracted DNA was amplified with validated probes (assay ID: C__11975651–10, C__850486–20, C__12005959–10, and C__3068176–10, respectively; Applied Biosystems). The *TS* VNTR polymorphism was defined by PCR using 5′-CGT GGC TCC TGC GTT TCC-3′ and 5′-GAG CCG GCC ACA GGC AT-3′ primers. In our laboratory, the quality of genotyping was routinely assessed statistically using the Hardy–Weinberg test. When allelic distributions for controls departed from the Hardy–Weinberg frequency, genotyping was assessed using DNA sequencing.

Intake assessment for folate and other nutrients. The consumption of folate and other nutrients were determined using a food frequency questionnaire (FFQ), described in detail elsewhere.[32,33] Briefly, the FFQ consisted of 47 single food items with frequencies in the following eight categories: never or seldom, 1–3 times/month, 1–2 times/week, 3–4 times/week, 5–6 times/week, once/day, twice/day, and 3+ times/day. We estimated the average daily intake of nutrients by multiplying the food intake (in g) by the nutrient content per 100 g of food as listed in standard tables of food composition.[34–36] Consumption of folate and other vitamins from supplements was not able to be considered in total consumption because the questionnaire for multivitamins was not quantitative. Energy-adjusted intake of nutrients was calculated using the residual method.[37] The FFQ was validated by referring to a 3-day weighed dietary record as a standard, which showed reproducibility and validity to be acceptable.[38] The deattenuated correlation coefficients for energy-adjusted intakes of folate were 0.36 in men and 0.38 in women.

Consumption of tobacco and alcohol. Cumulative smoking dose was evaluated as pack-years, the product of the number of packs consumed per day and years of smoking. Smoking habit was entered under the following four categories: never, former, current smokers of <40 pack-years, and current smokers of ≥40 pack-years. Former smokers were defined as those who quit smoking at least 1 year before the survey. Consumption of each type of beverage (Japanese sake, beer, shochu, whiskey and wine) was determined by the average number of drinks per day, which was then converted into a Japanese sake (rice wine) equivalent. One drink equates to one 'go' (180 mL) of Japanese sake, which contains 23 g ethanol, equivalent to one large bottle (633 mL) of beer, two shots (60 mL) of whiskey and two and a half glasses of wine (200 mL). One drink of 'Shochu' (distilled spirit), which contains 25% ethanol, was rated as 108 mL. Total amount of alcohol consumption was estimated as the summarized amount of pure alcohol consumption (g/drink) of Japanese sake, beer, shochu, whiskey and wine among current regular drinkers. Drinking habit was entered in the following four categories: never, former, current moderate and heavy drinkers. Heavy drinkers were defined as those currently drinking alcoholic beverages 5 days or more per week in a daily amount of 46 g (two Japanese drinks) or more, whereas moderate drinkers were defined as those currently consuming less frequently than 5 days per week, in lower amounts, or both. Former drinkers were defined as those who quit drinking at least 1 year before the survey.

Statistical analysis. To assess the strength of the associations between polymorphic genes involved in folate metabolism and risk of HNSCC, odd ratios (OR) with 95% confidence intervals (CI) were estimated using age- and sex-matched conditional logistic models adjusted for potential confounders. Folate and other nutrient intakes were categorized into three groups as follows: first, second, and third tertiles of dietary intake among controls. Potential confounders considered in the multivariate analyses were age, sex, smoking habit (never smokers, former smokers, current smokers of <40 pack-years or current smokers of ≥40 pack-years), drinking habit (never drinkers, former drinkers, moderate drinkers or heavy drinkers), multivitamin use (at least once per week for 1 year or longer: yes or no), total non-alcohol energy intake (as a continuous variable), dietary carotene intake (μg/day, tertiles), dietary vitamin C intake (mg/day, tertiles), dietary vitamin E intake (mg/day, tertiles), dietary folate intake (μg/day, tertiles), dental brushing (at least once/day: yes or no),[39] and referral pattern (self recommendation, recommendation by family or friends, referral by physicians,

Table 1. Characteristics of cases and controls

Characteristic	No. cases (n = 237)	No. controls (n = 711)	P-value
Age (years)			
<50	53 (22.4%)	138 (19.4%)	
50–59	67 (28.3%)	217 (30.5%)	
60–69	69 (29.1%)	213 (30.0%)	
70–79	48 (20.3%)	143 (20.1%)	
Mean age (SD)	57.9 (12.4)	58.4 (12.0)	
Sex			
Male	188 (79.3%)	564 (79.3%)	
Female	49 (20.7%)	147 (20.7%)	
Drinking			
Never	56 (23.6%)	235 (33.1%)	
Former[†]	14 (5.9%)	37 (5.2%)	
Current			
Moderate[‡]	96 (40.5%)	345 (48.5%)	
Heavy[§]	62 (26.2%)	83 (11.7%)	<0.01
Unknown	9 (3.8%)	11 (1.5%)	
Smoking			
Never	56 (23.6%)	256 (36.0%)	
Former[†]	64 (27.0%)	210 (29.5%)	
Current (pack years)			
0–39	52 (21.9%)	134 (18.8%)	
≥40	62 (26.2%)	108 (15.2%)	<0.01
Unknown	3 (1.3%)	3 (0.4%)	
Mean total energy (kcal/day) (SD)	1652.0 (381.6)	1689.1 (333.0)	0.20
Mean total non-alcohol energy (kcal/day) (SD)	1545.1 (358.4)	1622.5 (327.1)	<0.01
Carotene (mean ± SD, μg/day)			
Tertile 1 (1829.8 ± 209.8)	104 (43.9%)	236 (33.2%)	
Tertile 2 (2667.3 ± 314.0)	73 (30.8%)	236 (33.2%)	
Tertile 3 (4394.3 ± 1317.4)	55 (23.2%)	236 (33.2%)	<0.01
Unknown	5 (2.1%)	3 (0.4%)	
Vitamin C (mean ± SD, mg/day)			
Tertile 1 (53.9 ± 9.7)	113 (47.7%)	237 (33.3%)	
Tertile 2 (82.5 ± 8.2)	72 (30.4%)	237 (33.3%)	
Tertile 3 (129.0 ± 32.9)	47 (19.8%)	236 (33.2%)	<0.01
Unknown	5 (2.1%)	1 (0.1%)	
Vitamin E (mean ± SD, total α-mg/day)			
Tertile 1 (3.9 ± 0.6)	96 (40.5%)	237 (33.3%)	
Tertile 2 (5.5 ± 0.5)	88 (37.1%)	237 (33.3%)	
Tertile 3 (8.1 ± 1.8)	50 (21.1%)	236 (33.2%)	<0.01
Unknown	3 (1.3%)	1 (0.1%)	
Folate (mean ± SD, μg/day)			
Tertile 1 (218.5 ± 31.5)	111 (46.8%)	237 (33.3%)	
Tertile 2 (305.3 ± 23.4)	67 (28.3%)	237 (33.3%)	
Tertile 3 (435.3 ± 91.7)	54 (22.8%)	236 (33.2%)	<0.01
Unknown	5 (2.1%)	1 (0.1%)	
Multivitamin use (at least once per week for 1 year or longer)			
Yes	32 (13.5%)	162 (22.8%)	
No	191 (80.6%)	511 (71.9%)	0.01
Unknown	14 (5.9%)	38 (5.3%)	
Dental brushing (at least once per day)			
Yes	227 (95.8%)	703 (98.9%)	
No	10 (4.2%)	8 (1.1%)	<0.01
Referral pattern to our hospital			
Self recommendation	26 (11.0%)	202 (28.4%)	
Recommendation by family or friends	19 (8.0%)	143 (20.1%)	
Referral by physicians	177 (74.7%)	211 (29.7%)	
Secondary screening	11 (4.6%)	148 (20.8%)	
Others	0 (0%)	4 (0.6%)	<0.01
Unknown	4 (1.7%)	3 (0.4%)	

[†]Former smokers and drinkers were defined as subjects who had quit smoking and drinking at least 1 year previously. [‡]Moderate drinker means <46 g ethanol/drink and/or <5 days/week. [§]Heavy drinker means ≥46 g ethanol/drink and ≥5 days/week.

Table 2. Adjusted odds ratios (OR) relating associations with dietary folate intake for risk of head and neck squamous cell carcinoma

Folate intake (μg/day)	Cases/controls	OR† (95% CI)	P-value
Tertile 1 (133.0–265.0)	111/237	1.00 (reference)	
Tertile 2 (265.9–345.2)	67/237	0.55 (0.34–0.88)	
Tertile 3 (345.2–975.1)	54/236	0.53 (0.32–0.89)	0.02
Unknown	5/1		

†OR was matched for age and sex and adjusted for drinking habit, smoking habit, non-alcohol energy intake, multivitamin use, dental brushing and referral pattern to our hospital. CI, confidence interval.

secondary screening, or others). The internal validity of this hospital-based study is a potential threat to causal inference in this population. We used non-cancer patients at our hospital as controls, given the likelihood that our cases arose within this population base. To account for the difference between cases and controls, we adjusted for referral pattern to our hospital. Missing values for each covariate were excluded in the logistic model. Differences in categorized demographic variables and mean values between the cases and controls were tested using the Mantel–Haenszel method. Accordance with the Hardy–Weinberg equilibrium was checked for controls using the χ^2-test and the exact P-value was used to assess any discrepancies between genotype and allele frequencies. As a basis for the trend test, the median values of each tertile of folate intake consumption were included in the model. Gene–environment interactions between drinking and smoking habit and folate intake and genotypes in each polymorphism were evaluated under the multiplicative assumption. Products of scores for genotype (0, referent allele; and 1, non-referent allele) and drinking habit (0, never drinker; 1, moderate drinker; and 2, heavy drinker), smoking habit (0, never smoker; 1, current smoker of <40 pack-years; and 2, current smokers of ≥40 pack-years) and folate intake (0, tertile 1; and 1, tertile 2 + 3) were included as interaction terms. A P-value less than 0.05 was

considered statistically significant. All analyses were carried out using STATA version 9 (Stata Corp., College Station, TX, USA).

Results

Data from 237 HNSCC cases, comprising 119 (50%) oral cancers, 86 (36%) pharyngeal cancers and 32 (14%) laryngeal cancers, and 711 controls were available for analysis. Table 1 shows the distribution of cases and controls by background characteristics. Age and sex were matched. Heavy drinkers comprised 26.2% of the cases and this was significantly higher than for the controls, at 11.7%. Smoking habits also differed to a large extent between cases and controls, with 26.2 and 15.2% smokers of <40 pack-years or ≥40 pack-years, respectively. Total non-alcohol energy intake was lower in the case group. Lower intakes of dietary antioxidant vitamins, such as carotene, vitamins C and E, and folate were found among the cases. Multivitamin supplementation was more prevalent in the controls. The proportion of subjects without dental brushing at least once per day was significantly higher in the case group, although the number of subjects was low. With regard to referral pattern, referral by physicians was more frequent, whereas self recommendation, recommendation by family or friends and secondary screening were less common among the case group than the control group.

Table 2 shows the impact of dietary folate consumption on HNSCC risk. A significant inverse association was observed (trend $P = 0.02$).

Table 3 shows genotype distributions for *MTHFR*, *MTR*, *MTRR* and *TS*, and their OR and 95% CI for HNSCC risk. The genotype frequencies for all of the polymorphisms were in accordance with the Hardy–Weinberg law in controls: *MTHFR* C677T ($P = 0.29$), *MTHFR* A1298C ($P = 0.57$), *MTR* A2756G ($P = 0.87$), *MTRR* A66G ($P = 0.38$) and *TS* VNTR ($P = 0.51$). None of the polymorphisms showed any significant impact on HNSCC risk by genotype alone. When two polymorphisms were evaluated together (*MTHFR* C677T and *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G), no significant association with HNSCC risk was found (data not shown).

Table 3. *MTHFR*, *MTR*, *MTRR* and *TS* genotype distributions, and odds ratios (OR) for head and neck squamous cell carcinoma

Polymorphism	No. cases	No. controls	OR1† (95% CI)	P-value	OR2‡ (95% CI)	P-value
MTHFR (C677T)						
CC	88 (37.1%)	252 (35.4%)	1.00 (reference)		1.00 (reference)	
CT	113 (47.7%)	331 (46.6%)	0.98 (0.71–1.36)		0.98 (0.63–1.52)	
TT	36 (15.2%)	128 (18.0%)	0.80 (0.51–1.25)	0.39	0.77 (0.42–1.41)	0.46
MTHFR (A1298C)						
AA	149 (62.9%)	453 (63.7%)	1.00 (reference)		1.00 (reference)	
AC	79 (33.3%)	222 (31.2%)	1.09 (0.78–1.51)		1.07 (0.69–1.66)	
CC	8 (3.4%)	31 (4.4%)	0.79 (0.36–1.75)	0.96	0.82 (0.28–2.46)	0.97
UK§	1 (0.4%)	5 (0.7%)				
MTR (A2756G)						
AA	151 (63.7%)	496 (69.8%)	1.00 (reference)		1.00 (reference)	
AG	75 (31.6%)	195 (27.4%)	1.26 (0.91–1.73)		1.18 (0.76–1.83)	
GG	11 (4.6%)	20 (2.8%)	1.83 (0.84–3.97)	0.06	1.30 (0.49–3.44)	0.38
MTRR (A66G)						
AA	108 (45.6%)	332 (46.7%)	1.00 (reference)		1.00 (reference)	
AG	100 (42.2%)	315 (44.3%)	0.97 (0.71–1.33)		0.85 (0.55–1.31)	
GG	29 (12.2%)	64 (9.0%)	1.39 (0.85–2.26)	0.38	1.25 (0.64–2.44)	0.89
TS VNTR						
Non-2R/Non-2R	168 (70.9%)	502 (70.6%)	1.00 (reference)		1.00 (reference)	
2R/Non-2R	64 (27.0%)	188 (26.4%)	1.02 (0.73–1.42)		1.14 (0.72–1.78)	
2R/2R	5 (2.1%)	21 (3.0%)	0.71 (0.26–1.91)	0.78	0.66 (0.15–2.90)	0.84

†OR1 was matched for age and sex. ‡OR2 was matched for age and sex and adjusted for drinking habit, smoking habit, non-alcohol energy intake, carotene intake, vitamin C intake, vitamin E intake, folate intake, multivitamin use, dental brushing and referral pattern to our hospital. §UK denotes genotype unknown. CI, confidence interval.

Table 4. Interaction between *MTHFR*, *MTR*, *MTRR* or *TS* polymorphisms and drinking and smoking habit for head and neck squamous cell carcinoma risk

Polymorphism	Drinking habit						Interaction $P^{\S}$	Smoking habit						Interaction $P^{\S}$
	Never drinker		Moderate drinker[‡]		Heavy drinker[‡]			Never smoker		Current smoker pack years 0–39		Current smoker pack years ≥ 40		
	Cases/controls	OR[†] (95% CI)	Cases/controls	OR[†] (95% CI)	Cases/controls	OR[†] (95% CI)		Cases/controls	OR[†] (95% CI)	Cases/controls	OR[†] (95% CI)	Cases/controls	OR[†] (95% CI)	
MTHFR (C677T)														
CC + CT	45/193	1.00 (ref.)	85/286	1.48 (0.83–2.62)	57/68	4.41 (2.06–9.44)		48/211	1.00 (ref.)	44/119	0.62 (0.27–1.43)	52/82	1.57 (0.63–3.88)	
TT	11/42	1.94 (0.73–5.12)	11/59	0.90 (0.34–2.38)	5/15	1.61 (0.37–7.02)	0.04	8/45	1.61 (0.51–5.01)	8/15	0.98 (0.24–3.93)	10/26	1.02 (0.26–3.99)	0.30
MTHFR (A1298C)														
AA + AC	55/223	1.00 (ref.)	91/329	1.11 (0.64–1.92)	60/80	3.15 (1.57–6.31)		55/242	1.00 (ref.)	52/127	0.57 (0.25–1.27)	59/102	1.23 (0.50–3.03)	
CC	1/11	0.14 (0.01–1.46)	4/14	1.09 (0.22–5.26)	2/3	5.92 (0.17–206.63)	0.13	1/12	0.07 (0.01–0.97)	0/5	NA[¶]	3/6	2.75 (0.26–28.77)	0.06
MTR (A2756G)														
AA + AG	51/230	1.00 (ref.)	91/337	1.20 (0.70–2.05)	61/77	3.76 (1.84–7.69)		54/251	1.00 (ref.)	51/130	0.66 (0.29–1.48)	59/106	1.35 (0.55–3.29)	
GG	5/5	4.49 (0.72–28.19)	5/8	2.01 (0.47–8.60)	1/6	0.62 (0.05–7.29)	0.04	2/5	0.74 (0.08–6.64)	1/4	0.26 (0.02–4.16)	3/2	2.67 (0.07–106.29)	0.75
MTRR (A66G)														
AA + AG	51/213	1.00 (ref.)	86/313	1.09 (0.63–1.87)	52/78	2.61 (1.26–5.39)		49/232	1.00 (ref.)	45/123	0.68 (0.30–1.56)	54/99	1.52 (0.61–3.81)	
GG	5/22	0.51 (0.14–1.91)	10/32	1.76 (0.62–4.95)	10/5	12.39 (2.51–61.25)	0.03	7/24	1.68 (0.47–5.99)	7/11	0.79 (0.15–4.13)	8/9	1.41 (0.31–6.40)	0.55
TS VNTR														
Non-2R/Non-2R	37/172	1.00 (ref.)	70/241	1.61 (0.86–3.00)	44/55	4.23 (1.84–9.73)		41/184	1.00 (ref.)	37/93	0.77 (0.32–1.84)	47/77	1.98 (0.75–5.23)	
2R/Non-2R + 2R/2R	19/63	1.92 (0.87–4.23)	26/104	1.31 (0.61–2.81)	18/28	4.02 (1.43–11.34)	0.22	15/72	1.86 (0.77–4.53)	15/41	0.62 (0.17–2.31)	15/31	0.95 (0.26–3.47)	0.08

[†]Odds ratio (OR) was matched for age and sex and adjusted for drinking habit, smoking habit, non-alcohol energy intake, carotene intake, vitamin C intake, vitamin E intake, folate intake, multivitamin use, dental brushing, and referral pattern to our hospital. [‡]Moderate drinker means <46 g ethanol/drink and/or <5 days/week. Heavy drinker means ≥46 g ethanol/drink and ≥5 days/week. [§]Interactions were modeled as a product of drinking habit in score (0, never; 1, moderate drinker; and 2, heavy drinker), smoking habit in score (0, never; 1, 0–39 pack years; and 2, pack years≥ 40) and genotype in score. [¶]NA indicates that OR was not available because of insufficient data. CI, confidence interval.

Table 5. Interaction between *MTHFR*, *MTR*, *MTRR* or *TS* polymorphisms and folate intake for head and neck squamous cell carcinoma risk

| Polymorphism | Folate intake | | | | Interaction *P*[‡] |
| | Tertile 1 (133.0–264.7 µg/day) | | Tertile 2 + 3 (264.8–975.1 µg/day) | | |
	Cases/ controls	OR[†] (95% CI)	Cases/ controls	OR[†] (95% CI)	
MTHFR (C677T)					
CC + CT	93/203	1.00 (reference)	104/379	1.00 (0.54–1.79)	
TT	18/33	1.40 (0.58–3.21)	17/95	0.51 (0.23–1.15)	0.10
MTHFR (A1298C)					
AA + AC	105/224	1.00 (reference)	118/450	0.80 (0.46–1.39)	
CC	5/10	0.97 (0.21–4.46)	3/21	0.70 (0.16–3.12)	0.92
MTR (A2756G)					
AA + AG	105/227	1.00 (reference)	116/463	0.80 (0.46–1.39)	
GG	6/9	1.26 (0.34–4.70)	5/11	1.14 (0.25–5.20)	0.90
MTRR (A66G)					
AA + AG	99/218	1.00 (reference)	105/428	0.81 (0.46–1.43)	
GG	12/18	1.64 (0.59–4.59)	16/46	0.96 (0.39–2.32)	0.61
TS VNTR					
Non-2R/Non-2R	82/168	1.00 (reference)	83/333	0.77 (0.42–1.42)	
2R/Non-2R + 2R/2R	29/68	1.03 (0.51–2.10)	38/141	0.88 (0.43–1.80)	0.82

[†]Odds ratio (OR) was matched for age and sex and adjusted for drinking habit, smoking habit, non-alcohol energy intake, carotene intake, vitamin C intake, vitamin E intake, multivitamin use, dental brushing and referral pattern to our hospital. [‡]Interaction was modeled as a product of folate intake in score (0, tertile 1; and 1, tertile 2 + 3) and genotype in score. CI, confidence interval.

Table 4 summarizes interactions between polymorphisms and drinking and smoking habits. There were interactions between drinking habit and *MTHFR* C677T (*P* = 0.04), *MTR* A2756G (*P* = 0.04) and *MTRR* A66G (*P* = 0.03). Among subjects with the *MTHFR* 677 TT or the *MTR* 2756 GG genotypes, OR were not increased in heavy drinkers compared with never drinkers, whereas the GG genotype of *MTRR* had a more increased risk among heavy drinkers compared with AA/AG genotype (OR = 12.39, 95% CI 2.51–61.25). No obvious associations were observed for the *MTHFR* A1298C and *TS* polymorphisms regarding combinations with alcohol drinking. Associations between polymorphisms examined and smoking habit were less clear.

Table 5 shows data for combinations of the polymorphisms and folate intake with regard to HNSCC risk. There was no clear interaction between folate consumption and these polymorphisms, but a non-significantly decreased risk of HNSCC was found among those reporting high folate intakes with the *MTHFR* 677TT genotype.

Discussion

The results of the present study of associations between folate intake or one-carbon metabolism-related gene polymorphisms (*MTHFR* C677T and A1298C, *MTR* A2756G, *MTRR* A66G and *TS* VNTR) and HNSCC risk, combined with alcohol drinking, tobacco smoking and intake of folate, suggest that: (1) intake of folate is inversely associated with HNSCC risk; (2) none of the polymorphisms independently has any significant effect on HNSCC risk; and (3) *MTHFR* C677T, *MTR* A2756G and *MTRR* A66G may modify HNSCC risk by alcohol consumption.

Folate deficiency may increase the risk of cancer by inducing an imbalance in DNA precursors, leading to modified DNA synthesis and repair. It can also alter normal DNA methylation, which may contribute to loss of normal control of protooncogene expression.[12–14] Numerous studies have shown a reduced risk of HNSCC with increased fruit or vegetable consumption,[3,4] but few studies have examined the role of folate specifically. In two studies, folate intake was negatively associated with risk of head

and neck cancer.[8,9] Our finding, which adjusted for potential confounders, was in accordance with these results.

A high plasma level of folate has been found to decrease the risk of HNSCC in some case-control studies.[10,11] Functional polymorphisms of genes playing an important role in folate metabolism contribute to alterations in plasma levels of folate.[19] It is therefore plausible that they have a substantial impact on risk; however, few studies have previously examined the relationships between these polymorphisms and HNSCC risk. Hospital-based case-control studies in the USA showed a decreased risk with *MTHFR* 1298 AC/CC and *MTRR* AA genotypes, an increased risk with *MTR* AG/GG genotypes and no association with *MTHFR* C677T and *TS* polymorphisms.[25–27] A population-based case-control study in Puerto Rico reported that individuals with the *MTHFR* 677 TT genotype have a non-significantly lower risk of developing oral cancer.[28]

We failed to find any association between any of the folate metabolism-related polymorphisms alone and HNSCC risk; however, combined analysis suggested that the polymorphisms can modify the relationship between alcohol consumption and HNSCC risk. Risk reduction with drinking was seen among individuals with the *MTHFR* 677TT and *MTR* 2756 GG genotypes, whereas an increased risk was found for the *MTRR* 66 GG genotype. To our knowledge, the significant interaction between drinking status and these polymorphisms on HNSCC risk has not been reported previously, although the results from the combined analyses must be treated with caution due to reduced numbers of observations in the subgroups.

We found the *MTRR* 66GG genotype to be a significant HNSCC risk factor among heavy drinkers compared with the AA + AG genotype. Although the functional effects have not been fully established, the G allele of *MTRR* is considered to decrease the enzyme activity compared with the A allele.[40] Subjects with *MTRR* 66GG may have reduced methionine levels compared with those who had other genotypes. However, MTR, which is maintained by MTRR, is responsible for the remethylation of homocysteine to methionine. Ethanol has been reported to reduce mRNA and activity of MTR.[41] These findings suggest

that hypomethylation of DNA, which results in a predisposition to cancer, occurs among heavy drinkers with *MTRR* 66GG.[42] This speculation is consistent with our results, but further studies on the underlying mechanisms of the *MTRR* polymorphism are warranted.

A previous study showed a significant interaction between smoking status and the polymorphisms for HNSCC risk,[26] but our results did not confirm the association. In addition, because risk of other site neoplasia associated with the folate metabolism-related polymorphisms has been shown previously to differ by folate intake status,[19,43,44] we stratified the analyses of genotype combinations by dietary intakes of folate. Although no clear interaction was seen in the present study, the *MTHFR* 677 TT genotype showed a non-significantly reduced risk of HNSCC among subjects with an adequate intake of folate. This trend is consistent with previous studies.[19,43,44]

Several potential limitations of the present study warrant consideration. First, because this was a hospital-based case-control study, the source population from which cases arise may differ from that for controls. To account for the difference of background between cases and controls, we adjusted for referral pattern to our hospital. Second, as with other case-control studies, this study may suffer from recall bias. Although the questionnaires were completed before the diagnosis in our hospital, in some cases patients referred to the hospital might have known the diagnosis. Third, we used a self-administered questionnaire to evaluate folate intake. Data obtained from the FFQ may not reflect intake as accurately as those from other methods, such as biological markers. Nevertheless, the reproducibility and validity of the FFQ were acceptable.[38] We could not consider consumption of folate from supplements in total consumption, but the proportion of folate-containing supplement users is very low in Japan (0.1%).[45] Last, our study had a modest sample size, and these influences may reach significance in a larger study.

In conclusion, our case-control study suggested gene–environment interactions between alcohol drinking and the *MTHFR* C677T and *MTRR* A66G polymorphisms for HNSCC risk in the Japanese population.

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