Associations Between *MTHFR* Polymorphisms and the Risk of Potentially Malignant Oral Disorders

THOMAS SENGHORE¹, YU-FENG LI², FUNG-CHANG SUNG³, MING-HSUI TSAI⁴,
CHUN-HUNG HUA⁴, CHIU-SHONG LIU⁵, MIN-FENG HUNG⁶ and CHIH-CHING YEH^{1,3*}

¹School of Public Health, College of Public Health, Taipei Medical University, Taipei, Taiwan, R.O.C.;

²Graduate Institute of Biostatistics, Departments of ³Public Health, and

⁶Health Risk Management, College of Public Health,

China Medical University, Taichung, Taiwan, R.O.C.;

Departments of ⁴Otolaryngology, and ⁵Family Medicine,
China Medical University Hospital, Taichung, Taiwan, R.O.C.

Abstract. Aim: The study aimed to investigate the role of two polymorphisms of methylenetetrahydrofolate reductase (*MTHFR*), C677T and A1298C, in the risk of potentially malignant oral disorders (PMODs). Materials and Methods: Genotypes of the *MTHFR* C677T and A1298C polymorphisms were determined using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) for 224 PMOD cases and 485 age-matched controls. Results: The C677T T allele-carrying genotypes were significantly associated with a decreased risk of PMODs [odds ratio (OR)=0.62, 95% confidence interval (CI)=0.44-0.86]. Haplotype analysis also indicated that the 677T/1298A haplotype was associated with a decreased risk of PMODs (OR=0.56, 95%CI=0.40-0.80). No significant interaction was observed between *MTHFR* polymorphisms and lifestyle factors. Conclusion: Our findings suggest that the T-allele-carrying *MTHFR* C677T genotype or haplotype may reduce the risk of PMODs. However, these observations require further confirmation using larger samples.

Oral carcinoma (OC) remains a significant public health burden, with a high incidence and mortality globally (1). In Taiwan, OC is a leading cause of cancer morbidity and the fourth most-common cause of cancer mortality among men (2). Potentially malignant oral disorders (PMODs) that include leukoplakia, erythroplakia, and submucosal fibrosis,

have a very high rate of transformation to OC, ranging from 4-13% depending on the follow-up time and subtype (3, 4). Although individuals who have habits such as cigarette smoking, and alcohol or betel nut consumption are at risk for PMOD development, only a small fraction of these individuals actually develop such a disorder, suggesting that genetics may be implicated in the etiology (5).

The methylenetetrahydrofolate reductase (*MTHFR*) gene encodes a cytoplasmic flavoenzyme that channels the distribution of folate by regulating the balance between cellular methylation and nucleic acid synthesis (6), thus promoting DNA stability. Genetic variations of *MTHFR* have been found to significantly influence many malignancies, including oral squamous cell carcinoma (7). Two widely studied polymorphisms in *MTHFR* (C667T and A1298C) were found to have a reduced enzymatic activity (8), which results in substrate accumulation in the *MTHFR* pathway. This causes a disruption in nucleic acid synthesis and a decrease in the availability of methyl groups for cellular biochemical processes (9). Carriers of the 667T allele have about 35-70% lower enzymatic activity (10), while the 1298CC genotype has about 40% lower enzymatic activity than do individuals with the AA wild-type genotype (11).

Given the link between polymorphisms of *MTHFR* C677T and A1298C with a reduced level of genomic DNA methylation, and the influence of *MTHFR* C667T and A1298C on the risk of OCs (12), we attempted to investigate the association between *MTHFR* polymorphisms and the risk of PMODs.

Materials and Methods

Study population. Participants were recruited from a male penitentiary in Taiwan. Of the estimated 4000 inmates, about 76% consented and were eligible to participate in the study. Irrespective

Correspondence to: Professor Chih-Ching Yeh, Ph.D., MSPH, School of Public Health, Taipei Medical University, 250 Wu-Hsing Street, Taipei 11031, Taiwan, R.O.C. Tel: 886 27361661 ext. 6534, Fax: +886 27384831, e-mail: ccyeh@tmu.edu.tw

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of their eligibility status, all inmates underwent a complete oral examination by two experienced otolaryngologists, as per World Health Organization (WHO) criteria (13). All cases were diagnosed as leukoplakia, erythroplakia, submucous fibrosis, oral cancer, or as having other minor oral conditions. Only cases diagnosed as leukoplakia or submucous fibrosis were included in this analysis. Participants with normal oral screening results were used as controls, matched for age.

All eligible participants provided information on their sociodemographic, lifestyle, and medical history through a detailed structured self-administered questionnaire. Sociodemographic and lifestyle factors included cigarette smoking, alcohol consumption, and betel nut chewing, all of which were categorized into three groups as: non-users, current users (those who had used for more than 1 year), and former users (having stopped using continuously for at least the previous 6 months). Total cigarettes consumed were estimated in pack-years as daily consumption multiplied by the duration (in years). The personal medical history, including family history of first-degree relatives only, was also collected. Height and weight were measured, and the body-mass index (BMI) was calculated as weight/height² (kg/m²). All participants gave their written informed consent to participation after a detailed description of the research purpose, and the study was approved by the China Medical University Ethics Review Community and the Institutional Review Board at China Medical University Hospital (IRB no: DMR96-IRB-085).

Single-nucleotide polymorphism (SNP) selection and genotyping. Two functional SNPs in the *MTHFR* gene were genotyped in this study. These polymorphisms were reported to affect either the expression or function of their host gene or are associated with a risk of malignancy (14, 15).

Genotyping was performed using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). All PCRs were performed in a 20- μ l final volume with 5 pM of each primer, 50 ng genomic DNA, 1.5 mM MgCl₂, 200 μ M dNTPs, and 1.0 units of Taq DNA polymerase in buffer provided by the manufacturer (Fermentas, Glen Burnie, MD, USA). A Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) was used to perform the amplification. Earlier, from buffer coat cells, genomic DNA was extracted using a DNA extraction kit (Qiagen, Chatsworth, CA, USA).

For the *MTHFR* C677T (rs1801133) polymorphism, primer sequences used were as follows: 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' (forward primer) and 5'-AGG ACG GTG CGG TGA GAG TG-3' (reverse primer). The PCR amplification procedure consisted of 2 min of denaturing at 94°C followed by 30 cycles of 30 s at 94°C and 30 s at 72°C. The 198-bp PCR product was digested in *Hinf*I (New England BioLabs, Beverly, MA, USA): The T allele was cut into 175- and 23-bp fragments and the C allele was not digested. In the case of the *MTHFR* A1298C (rs1801131) polymorphism, the following primers were used: 5'-ATG TGG GGG GAG GAG CTG AC-3' (forward primer) and 5'-GTC TCC CAA CTT ACC CTT CTC CC-3' (reverse primer). The PCR cycling conditions included a 5-min denaturing at 92°C, then 35 cycles of 60 s at 92°C, followed by 30 s at 60.5°C and finally 30 s at 72°C. The 241-bp PCR product was digested with *Mbo*II (Fermentas): The A allele was cut into 204- and 37-bp fragments and the C allele was not digested. Ten percent of genotype determinations were carried out twice in independent experiments, with 100% concordance for both SNPs.

Table I. Comparisons of characteristics between cases with potentially malignant oral disorders and healthy controls.

Variable ^a	Controls N=485	Cases N=224	p-Value ^c
Mean age (SD), years	38.1 (9.1)	38.8 (9.2)	0.403
BMI (SD), kg/m ²	23.8 (3.0)	24.6 (3.7)	0.003
Educational level, n (%)			0.043
Below elementary school	80 (16.5)	43 (19.6)	
Junior high school	216 (44.6)	113 (51.4)	
Above senior high school	188 (38.8)	64 (29.1)	
Cigarette smoking, n (%)			0.084
Never	24 (5.0)	5 (2.2)	
Current smoker	424 (87.4)	208 (92.9)	
Ex-smoker	37 (7.6)	11 (4.9)	
Pack-years of cigarettes ^b , n (%)			0.315
≤ 7.5	141 (29.1)	51 (22.8)	
7.6-13.0	115 (23.7)	54 (24.1)	
13.1-20.0	123 (25.4)	61 (27.2)	
>20.1	106 (21.9)	58 (25.9)	
Alcohol consumption, n (%)			0.699
Never	175 (36.1)	74 (33.0)	
Current drinker	169 (34.9)	84 (37.5)	
Ex-drinker	141 (29.1)	66 (29.5)	
Betel nut chewing, n (%)			0.024
Never	166 (34.3)	54 (24.1)	
Current chewer	129 (26.7)	69 (30.8)	
Ex-chewer	189 (39.1)	101 (45.1)	
Tea consumption, n (%)			0.500
Never	135 (27.9)	57 (25.5)	
Current drinker	333 (68.8)	156 (69.6)	
Ex-drinker	16 (3.3)	11 (4.9)	
Family cancer history, n (%)			0.737
No	375 (77.5)	171 (76.3)	
Yes	109 (22.5)	53 (23.7)	

SD, Standard deviation; BMI, body-mass index. ^aNumbers may not sum to the total number due to missing data. ^bQuartile pack-years of cigarettes among the controls. ^cStudent's *t*-test or Chi-square test.

Statistical analysis. Pearson's Chi-squared and Student's *t*-test were used to assess participant characteristics. Hardy-Weinberg equilibrium (HWE) was tested using a goodness-of-fit chi-squared test. The PMOD risk was estimated as odds ratios (ORs) and 95% confidence intervals (CIs) using a multivariate logistic regression adjusted for educational level, BMI, cigarette smoking (pack-years), alcohol consumption, and betel nut chewing. Common variants were considered as the reference. Based on habit, participants with cigarette smoking status were dichotomized into a single binary variable by the median of 13 pack-years of cigarettes for the controls, with similar multivariate logistic regression models used to examine gene-habit interactions. The same analysis was performed for betel nut chewing and alcohol consumption, stratifying participants into never and ever having chewed betel nut or consumed alcohol. Finally, the haplotypes were constructed, and analyses were performed with the THESIAS program (JAVA version; INSERM U525, Paris, France). All analyses were two-sided, and a *p*-value of less than 0.05 was considered statistically

Table II. Associations between methylenetetrahydrofolate reductase (*MTHFR*) polymorphisms and the risk of potentially malignant oral disorders.

<i>MTHFR</i> genotype	Controls N=485 n (%)	All cases (N=224)			Leukoplakia (N=170)			Submucosal fibrosis (N=78)		
		n (%)	OR (95% CI) ^a	<i>p</i> -Value	n (%)	OR (95% CI) ^a	<i>p</i> -Value	n (%)	OR (95% CI) ^a	<i>p</i> -Value
C677T										
CC	256 (52.8)	145 (64.7)	1.00		107 (62.9)	1.00		57 (73.1)	1.00	
CT	193 (39.8)	70 (31.3)	0.66 (0.47-0.94)	0.022	55 (32.4)	0.71 (0.48-1.05)	0.088	20 (25.6)	0.48 (0.27-0.85)	0.011
TT	36 (7.4)	9 (4.0)	0.36 (0.15-0.83)	0.017	8 (4.7)	0.42 (0.17-1.05)	0.063	1 (1.3)	0.13 (0.02-0.96)	0.046
CC	256 (52.8)	145 (64.7)	1.00		107 (62.9)	1.00		57 (73.1)	1.00	
CT+TT	229 (47.2)	79 (36.3)	0.62 (0.44-0.86)	0.005	63 (37.1)	0.66 (0.46-0.97)	0.034	21 (26.9)	0.42 (0.24-0.74)	0.002
A1298C										
AA	286 (59.0)	121 (54.0)	1.00		86 (50.6)	1.00		47 (60.3)	1.00	
AC	166 (34.2)	88 (39.3)	1.26 (0.89-1.77)	0.193	71 (41.8)	1.40 (0.96-2.05)	0.080	25 (32.1)	0.96 (0.56-1.64)	0.869
CC	33 (6.8)	15 (6.7)	1.07 (0.55-2.07)	0.841	13 (7.7)	1.28 (0.63-2.59)	0.494	6 (7.7)	1.02 (0.39-2.70)	0.967
AA	286 (59.0)	121 (54.0)	1.00		86 (50.6)	1.00		47 (60.3)	1.00	
AC+CC	199 (41.0)	103 (46.0)	1.23 (0.88-1.70)	0.224	84 (49.4)	1.38 (0.96-1.99)	0.080	31 (39.7)	0.97 (0.58-1.61)	0.896

OR, Odds ratio; CI, confidence interval. ^aAdjusted for educational level, body-mass index, cigarette smoking (pack-years), alcohol consumption, and betel nut chewing.

significant. The SAS Statistical Package (vers. 9.4 for Windows; SAS Institute, Cary, NC, USA) was used for all analyses.

Results

In total, 224 PMOD cases and 485 cancer-free controls, matched by age were included in the study. Summary statistics of sociodemographic and lifestyle risk factors are shown in Table I. Statistically significant differences between the cases and controls were only observed in mean BMI, educational level, and betel nut chewing. Cases had a higher mean BMI (24.6 ± 3.7 vs. 23.8 ± 3.0 kg/m², $p=0.003$), a lower percentage with above senior secondary school education (29.1 vs. 38.8%, $p=0.043$), and a higher proportion of current and ex-betel nut chewers (30.8 and 45.1% vs. 26.7 and 39.1%, respectively, $p=0.024$) compared to controls.

Distributions of variants in both cases and controls were found to correspond to Hardy–Weinberg equilibrium. Heterozygous carriers of the C667T (OR=0.66, 95% CI=0.47-0.94, $p=0.022$) and T/T homozygous (OR=0.36, 95% CI=0.15-0.83, $p=0.017$) variants were at an overall decreased risk of PMOD compared to those with wild-type (CC), after adjusting for potential confounders. The C677T T-allele-carrying genotypes were significantly associated with a decreased risk of PMODs (OR=0.62, 95% CI=0.44-0.86, $p=0.005$). Further stratification by PMOD subtypes found similarly significantly reduced risk for submucosal fibrosis (OR=0.48, 95% CI=0.27-0.85, $p=0.011$ and OR=0.13, 95% CI=0.02-0.74, $p=0.046$ for CT and TT, respectively) but not for leukoplakia. A similar analysis was performed on A1298C genotypes, but no significant associations were found in either the overall or stratified analysis (Table II).

Joint effects of gene and lifestyle factors on PMOD risk are shown in Table III. Of the two SNPs evaluated, only the C677T genotype was still significantly associated with PMOD risk in the stratification analysis for all three risky habits; however, this interaction was not statistically significant. In the haplotype analysis, the C677T/A1298C TA haplotype had a significantly lower risk for PMODs overall ($p=0.001$), leukoplakia ($p=0.018$), and submucosal fibrosis ($p=0.002$) compared to the most common CA haplotype (Table IV).

Discussion

Considering that PMODs have a high transformation rate into OC, understanding the genetic susceptibility for PMOD may provide an opportunity for risk assessment of OC early in the disease course. In this study, we found that carriers of the CT and TT genotypes of C677T had about 1.5- and 2.7-fold, respectively, reduced risk for PMODs compared to those with wild-type CC genotype. The reduced risk manifested by these genotypes may be due to reduced activity of the corresponding enzymes that causes expanded intracellular pools of 5,10-MTHF. The subsequent substrate accumulation increases the availability of thymine, eventually enhancing DNA stability by sequestering 5, 10-MTHF for thymidine synthesis, which lowers the misincorporation of uracil into DNA and reduces global DNA methylation (16).

Many other studies also found protective effects of *MTHFR* variants on prostate and oral cancer in Asian populations (12, 17). Our results are in contrast with those of other studies which indicated an increased risk of OSCC in southeastern Iranians (7), and no association in southern Brazilians (18). These differences in findings might reflect

Table III. Associations between methylenetetrahydrofolate reductase (MTHFR) genotypes and risk of potentially malignant oral disorders stratified by cigarette smoking, betel nut use and alcohol consumption.

MTHFR genotype		Controls (N=485)	All cases (N=224)		Leukoplakia (N=170)		Submucosal fibrosis (N=78)	
		n	n	OR (95% CI)	n	OR (95% CI)	n	OR (95% CI)
C677T	Pack-years of cigarettes							
CC	≤13	132	64	1.00	43	1.00	29	1.00
CT+TT	≤13	124	41	0.70 (0.43-1.13) ^a	31	0.80 (0.46-1.40) ^a	13	0.53 (0.26-1.11) ^a
CC	>13	124	81	1.00	64	1.00	28	1.00
CT+TT	>13	105	38	0.50 (0.31-0.81) ^{a*}	32	0.53 (0.32-0.89) ^{a*}	8	0.29 (0.12-0.71) ^{a*}
<i>p</i> for interaction				0.33		0.24		0.40
C677T	Betel nut chewing							
CC	Never	75	34	1.00	28	1.00	7	1.00
CT+TT	Never	91	20	0.45 (0.23-0.87) ^{b*}	17	0.46 (0.23-0.93) ^{b*}	3	0.46 (0.11-1.99) ^b
CC	Ever	181	111	1.00	79	1.00	50	1.00
CT+TT	Ever	137	59	0.70 (0.47-1.04) ^b	46	0.78 (0.50-1.23) ^b	18	0.46 (0.25-0.85) ^{b*}
<i>p</i> for interaction				0.23		0.21		0.63
C677T	Alcohol consumption							
CC	Never	83	48	1.00	40	1.00	11	1.00
CT+TT	Never	92	26	0.49 (0.28-0.89) ^{c*}	20	0.47 (0.25-0.89) ^{c*}	7	0.64 (0.23-1.76) ^c
CC	Ever	173	97	1.00	67	1.00	46	1.00
CT+TT	Ever	137	53	0.71 (0.47-1.08) ^c	43	0.84 (0.52-1.35) ^c	14	0.36 (0.18-0.72) ^{c*}
<i>p</i> for interaction				0.36		0.17		0.39

OR, Odds ratio; CI, confidence interval. ^aAdjusted for educational level, body-mass index, alcohol consumption, and betel nut chewing. ^bAdjusted for educational level, body-mass index, alcohol consumption, and cigarette smoking (pack-years). ^cAdjusted for educational level, body-mass index, cigarette smoking (pack-years) and betel nut chewing. *Significantly different at *p*<0.05.

Table IV. Haplotype analysis of methylenetetrahydrofolate reductase (MTHFR) polymorphisms and risk of potentially malignant oral disorders.

C677T/ A1298C	Controls (N=485)	All cases (N=224)			Leukoplakia (N=170)			Submucosal fibrosis (N=78)		
	%	%	OR (95% CI) ^a	<i>p</i> -Value	%	OR (95% CI) ^a	<i>p</i> -Value	%	OR (95% CI) ^a	<i>p</i> -Value
CA	0.500	0.567	1.00		0.536	1.00		0.631	1.00	
CC	0.229	0.244	0.93 (0.69-1.26)	0.654	0.265	1.07 (0.78-1.49)	0.668	0.232	0.80 (0.50-1.30)	0.370
TA	0.261	0.167	0.56 (0.40-0.80)	0.001	0.175	0.62 (0.42-0.92)	0.018	0.129	0.40 (0.23-0.71)	0.002
TC	0.011	0.022	1.69 (0.55-5.17)	0.357	0.024	1.98 (0.60-6.51)	0.261	0.007	0.61 (0.06-6.07)	0.674

OR, Odds ratio; CI, confidence interval. ^aAdjusted for educational level, body-mass index, cigarette smoking (pack-years), alcohol consumption, and betel nut chewing. Among controls, C677T was in weak linkage disequilibrium with A1298C ($r^2=0.08$, $D'=-0.83$; $p<0.0001$).

differences in ethnicity and tumor types, and may be the result of small sample sizes. Other evidence implicates aberrant DNA methylation in promoting tumorigenesis by selective growth and transformation of cells resulting from hypermethylation of tumor-suppressor genes. However, the decreased activity of MTHFR associated with reduced global DNA methylation was proven in mouse models (19).

No significant interactions between lifestyle factors and the two SNPs with PMOD risk were observed, but those with MTHFR C677T T-allele-carrying genotype possessed a significantly reduced risk for overall PMODs, leukoplakia, and submucosal fibrosis which was modified by smoking.

Our findings are supported by similar studies in Taiwanese (12, 20). Evidence suggests that the C677T CC genotype may affect MTHFR activity (10, 11), especially in the presence of high folate levels which thereby influence its normal function (21). In the stratified analysis of betel nut and alcohol consumption, those with the T-allele-carrying genotype who never chewed betel nut or consumed alcohol had a little over a 2-fold decreased risk for overall PMODs and leukoplakia compared to their CC genotype counterparts. On the contrary, betel nut and alcohol consumers with C677T C allele-carrying genotypes had significantly reduced risk only for submucosal fibrosis. In the literature, findings

on alcohol and betel nut consumption modifying the effect on head and neck cancer are mixed. While one found no such effect with alcohol and betel nut use (12), another found an interaction or a decreased association in heavy/ever drinkers (22), and still another found an increased association in heavy drinkers with the CT and TT genotypes (23). Interactions between the C677T SNP and these two environmental factors on PMOD risk may be complicated and not only involve folate but also other factors of the folate metabolic pathway (24). The discrepancy in findings may be attributable to differences in racial origin, lifestyle, and dietary pattern in different populations.

In the haplotype analysis, we found the C677T/A1298C TA haplotype to be associated with a reduced risk for PMODs, suggesting that the T allele of C677T may play a leading role in the determination of PMOD risk. As previously reported, van der Put *et al.* found the combined heterozygote of C677T and A1298C to lead to an effect similar to that of the C677T TT genotype (11).

Our study has certain limitations. Firstly, there was a lack of information on dietary folate intake and plasma folate level. Secondly, participants included only males. Finally, the statistical power was low, especially in the stratification analysis. Despite these limitations, to our knowledge, no other study investigated the effects of *MTHFR* genotypes on PMOD risk. These observations may require further confirmation using larger samples.

Conclusion

Our findings suggest that the *MTHFR* 677 genotypes and C677T/A1298C haplotype with T-allele, but not the A1298C polymorphism, may be associated with a significantly reduced risk for PMODs in a Taiwanese population.

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