Methylenetetrahydrofolate Reductase (MTHFR) genotype, Smoking Habit, Metastasis and Oral Cancer in Taiwan

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Abstract. The aim of this study was to evaluate the association and interaction of genotypic polymorphism in *methylenetetrahydrofolate reductase (MTHFR)* with smoking habits and oral cancer in Taiwan. Two well-known polymorphic variants of *MTHFR*, C677T (rs1801133) and A1298C (rs1801131), were analyzed in association with oral cancer risk, and their joint effects with individual smoking habits on oral cancer risk were discussed. In total, 620 oral cancer patients and 620 non-cancer controls in central Taiwan were recruited and genotyped. The *MTHFR* C677T genotype, but not the A1298C, was differently distributed between the oral cancer and control groups. The T allele of *MTHFR* C677T was significantly more frequently found in controls than in oral cancer patients. Joint effects of smoking and *MTHFR* C677T genotype significantly affected oral cancer susceptibility. The *MTHFR* C677T CT and TT genotypes in association with smoking conferred lower odds ratios of 0.66 and 0.54 (95% confidence interval=0.49-0.82 and 0.39-0.86), for respectively. Those patients with *MTHFR* C677T CT and TT genotypes also had a lower risk of oral cancer metastasis. *MTHFR* C677T genotype may have joint effects with smoking on oral carcinogenesis, and may be a useful biomarker for prediction and prognosis of oral cancer.

Oral cancer is one of the most commonly diagnosed types of cancer worldwide (1-4), and has rapidly increasing incidence and mortality rates in Taiwan (5), with the highest incidence and mortality being recorded in central Taiwan. However, the genomic etiology of oral cancer is of great interest but remains unclear (5).

In recent years, the joint effects of environmental, lifestyle and genetic factors are receiving increased attention. Primary candidates for such interaction studies are those genes encoding enzymes related to the metabolism of established carcinogens. Methylenetetrahydrofolate reductase (*MTHFR*) is a key enzyme in folate metabolism and the potential protective effect of folate on cancer risk has been of research interest in the last decade (6). In humans, folate plays the fundamental role of providing methyl groups for deoxynucleoside synthesis and for intracellular methylation reactions (7), and low folate levels have been reported to lead to uracil misincorporation during DNA synthesis, chromosomal damage, DNA strand breaks, impaired DNA repair, and DNA hypomethylation (8).

Gene variants of key enzymes in the folate metabolism, such as the gene *MTHFR*, were suggested to be responsible for differences in folate levels and DNA methylation (9, 10). Previous investigations of *MTHFR* genetic variations focused on the catalytic domain and the two polymorphisms C677T and A1298C, which slightly change the enzymatic activity of the protein (10, 11). In the case of the C677T polymorphism, the cytosine base at position

number 677 changes to a thymidine base, which in turn affects the amino acid sequence from alanine to valine at position number 222. The *MTHFR* A1298C polymorphism is localized in the coding regulatory region domain (12). Studies investigating the *MTHFR* A1298C variant have found positive associations with colorectal cancer (13), breast cancer (14), acute lymphocytic leukemia (15), and childhood leukemia (16), but not with lung cancer risk (17).

A low level of genomic DNA methylation may also be linked to the *MTHFR* C677T and A1298C polymorphisms, which reduce MTHFR activity (18-20). The aim of the present study was to assess the overall effect of the *MTHFR* C677T and A1298C polymorphisms on oral cancer, the potential effect of the *MTHFR* genetic variants by personal habits, as well as their correlations with oral cancer prognosis.

Materials AND Methods

Study population and sample collection. Six hundred and twenty cancer patients diagnosed with oral cancer were recruited at the outpatient clinics of general surgery between 1994-2008 at the China Medical University Hospital, Taichung, Taiwan, Republic of China. The clinical characteristics of patients including histological details were all graded and defined by expert surgeons. All patients voluntarily participated, completed a self-administered questionnaire and provided peripheral blood samples. An equal number of non-cancer healthy volunteers as controls were selected by matching for age, gender and

habits after initial random sampling from the Health Examination Cohort of the hospital. The exclusion criteria of the control group included previous malignancy, metastasized cancer from other or unknown origin, and any familial or genetic disease. Both groups completed a short questionnaire which included their habits. Our study was approved by the Institutional Review Board of the China Medical University Hospital and written-informed consents were obtained from all participants.

Genotyping assays. Genomic DNA was prepared from peripheral blood leukocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan, R.O.C.) and further processed according to previous studies (17, 21-24). The polymerase chain reaction (PCR) cycling conditions were: one cycle at 94℃ for 5 min; 35 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s, and a final extension process at 72℃ for 10 min. The sequences of PCR primer pairs and the restriction enzyme for each DNA product are summarized in Table I.

Statistical analyses. Only data with both genotypic and clinical data (control/case=620/620) were selected for the final analysis. To ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, the deviation of the genotype frequencies of *MTHFR* SNPs in the controls from those expected under the Hardy-Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson's chi-square test was used to compare the distribution of the genotypes between cases and

controls. Data were considered as significant when the *P*-values were less than 0.05.

Results

The clinical characteristics of 620 oral cancer patients and the same number of non-cancer controls are summarized in Table II. These characteristics of patients and controls are all well matched since none of the differences between the groups were statistically significant ($P>0.05$) (Table II).

The frequencies of the genotypes for the *MTHFR* C677T and A1298C in controls and oral cancer patients are shown in Table III. The genotype distribution of the genetic polymorphisms of *MTHFR* C677T was significantly different between oral cancer and control groups ($P=0.0003$), while that for A1298C polymorphisms was not ($P>0.05$) (Table III). The frequencies of the alleles for *MTHFR* C677T and A1298C in controls and oral cancer patients are shown in Table IV. The C allele of the *MTHFR* C677T polymorphism was associated with oral cancer risk ($P=5.01\times10^{-5}$).

Since smoking, alcohol drinking and betel quid chewing are the predominant risk factors for oral cancer in Taiwan, the interaction between *MTHFR* genotype and individual habits was also analyzed by stratifying individual smoking status (Table V). We noticed that individuals with the CT or TT genotype of *MTHFR* C677T had a lower risk of oral cancer in the smoking group compared with those with CC, but this was not the similar case in the

non-smoking group. There was an interaction between *MTHFR* genotype and smoking status as regards oral cancer susceptibility, but no such relationship for alcohol drinking or betel quid chewing (data not shown).

The effects of *MTHFR* C677T genotype on oral cancer prognosis indices, recurrence and metastasis were also investigated (Table VI). The oral cancer patients with the CT or TT genotype of *MTHFR* C677T had a lower risk of metastasis compared with those with CC. As for oral cancer recurrence, no *MTHFR* C677T genotypic difference was found.

Discussion

In order to determine the role of *MTHFR* and to find potential biomarkers of oral cancer, in this study, we selected two well-known SNPs of the *MTHFR* gene and investigated their associations with oral cancer in a population of central Taiwan. The conclusion deduced from our data is that the *MTHFR* C677T T allele seems to be associated with a lower risk for oral cancer in Taiwan (Tables III and IV). These data are in agreement with the findings that the T allele appeared to potentially confer a lower risk (25-27), but are inconsistent with those finding it having no association with oral cancer (28-33). This may be caused by differences in ethnicity and sampling. Most importantly, our patient sample size was the largest (620) compared to all the previous studies mentioned above (from 50 to 583), and is the most representative and informative for Taiwan, where oral cancer incidence is highest in the

world.

We have further analyzed the joint effects of *MTHFR* C677T genotype and individual habits on oral cancer risk, including smoking, alcohol drinking and betel quid chewing. Interestingly, the interaction between *MTHFR* C677T genotype and cigarette smoking habit is clear, smokers with the CT and TT genotype have 0.66 and 0.54 lower risk (odds ratios) than smokers with CC genotype, which is not the case in the non-smokers (Table V). For alcohol drinking and betel quid chewing, there is no similar difference found. We have also analyzed the effect of *MTHFR* C677T genotype on oral cancer outcome, such as metastasis and recurrence, which has not been performed by other groups. We found that those patients with CT or TT genotypes of *MTHFR* C677T were at lower risk of metastasis, but there was no difference in recurrence compared with those with CC genotype (Table VI).

We propose that the C allele of C677T may affect MTHFR activity, slightly influencing its normal function (18-20). As individuals with the C allele(s) get older, the alteration towards carcinogenesis may accumulate *via* the decreasing functions of MTHFR. Cigarette smoking, a well-known cause of DNA damage, will release many DNA damage inducers into the respiratory system and cause DNA damage to cells. Therefore, in people who have a risky genetic variant, such as the C allele of C677T, and who also have a smoking habit, the joint effect of these factors may synergistically increase their oral cancer susceptibility. In oral cancer patients, the protective effects of CT or TT genotypes of *MTHFR* C677T may

influence the microenvironment near the oral cancer tumor site, resulting in preventing the tumor cells from metastasis.

Conclusions

In conclusion, this is the first study investigating the overall effects of the *MTHFR* genotypes on oral cancer, including their associations, their joint effect with individual habits, and their effect on oral cancer prognosis in Taiwan. The presence of the T allele of C677T was associated with a lower risk of oral cancer, and also a lower risk of metastasis. These data may be useful for developing MTHFR as an anticancer target, cancer biomarker and can aid in prognosis.

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Table I. The primer sequences, polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) conditions for *MTHFR* gene polymorphisms.

Polymorphism (location)	**Primer sequences (5' to 3')**	**Restriction enzyme**	**SNP sequence**	**DNA product (bp)**
C677T	F: TGA AGG AGA AGG TGT CTG CGG GA	*Hinf* I	C	198
(rs1801133)	R: AGG ACG GTG CGG TGA GAG TG		T	175 + 23
A1298C	F: GGGAGGAGCTGACCAGTGCAG	*Fnu4H* I	C	138
(rs1801131)	R: GGGGTCAGGCCAGGGGCAG		A	119 + 19

*F and R indicate forward and reverse primers, respectively.

Table II. Characteristics of oral cancer patients and controls.

Characteristic	Controls (N = 620)			Patients (N = 620)			*P*-value[a]
	N	%	Mean (SD)	N	%	Mean (SD)	
Age (years)			63.5 (8.5)			65.5 (9.7)	0.73
Gender							0.26
Male	572	92.3%		583	94.0%		
Female	48	7.7%		37	6.0%		
Indulgence							
Cigarette smokers	452	72.9%		463	74.6%		0.52
Areca chewers	390	62.9%		408	65.8%		0.31
Alcohol drinkers	432	69.6%		457	73.7%		0.13

[a] Based on chi-square test.

Table III. Distribution of *MTHFR* genotypes among oral cancer patient and control groups.

Genotype	Controls		Patients		*P*-value[a]
	N	%	N	%	
C677T rs1801133					**0.0003**
CC	322	51.9%	391	63.1%	
CT	236	38.1%	186	30.0%	
TT	62	10.0%	43	6.9%	
A1298C rs1801131					0.4455
AA	393	63.4%	407	65.6%	
AC	198	31.9%	192	31.0%	
CC	29	4.7%	21	3.4%	

[a] Based on chi-square test.

Table IV. *MTHFR* allelic frequencies among the oral cancer patient and control groups.

Allele	Controls		Patients		*P*-value[a]
	N	%	N	%	
C677T rs1801133					**5.01E-5**
Allele C	880	71.0%	968	78.1%	
Allele T	360	29.0%	272	21.9%	
A1298C rs1801131					0.2672
Allele A	984	79.4%	1006	81.1%	
Allele C	256	20.6%	234	18.9%	

[a] Based on chi-square test.

Table V. Distribution of *MTHFR* C677T genotype and oral cancer after stratification by smoking habit.

	Overall				Never smokers				Ever smokers			
C667T (rs1801133)	Controls	Cases	Adjusted[a]	*P*-value[c]	Controls	Cases	Adjusted[b]	*P*-value[c]	Controls	Cases	Adjusted[b]	*P*-value[c]
Genotype	N (%)	N (%)	OR (95% CI)		N (%)	N (%)	OR (95% CI)		N (%)	N (%)	OR (95% CI)	
CC	322 (51.9)	391 (63.1)	1.00 (Ref.)		81 (52.9)	88 (61.5)	1.00 (Ref.)		241 (51.6)	303 (63.5)	1.00 (Ref.)	
CT	236 (38.1)	186 (30.0)	0.64 (0.54-0.81)	0.0005	57 (37.3)	44 (30.8)	0.72 (0.41-1.65)	0.2085	179 (38.3)	142 (29.8)	0.66 (0.49-0.82)	0.0012
TT	62 (10.0)	43 (6.9)	0.56 (0.41-0.86)	0.0088	15 (9.8)	11 (7.7)	0.69 (0.36-1.53)	0.4034	47 (10.0)	32 (6.7)	0.54 (0.39-0.86)	0.0153

[a] Adjusted for age, gender and smoking (pack-years); [b] adjusted for age and gender. OR, Odds ratio; CI, confidence interval; Ref., reference; [c] based on chi-square test

Table VI. Interaction of *MTHFR* C677T genotype with oral cancer recurrence and metastasis.

Patient Status	*MTHFR* C677T			
	CC	CT+TT	*P*-value[a]	OR (95% CI) [b]
Recurrence status			0.6509	
No recurrence >5 years	357	212		1.00
Recurrence <5 years	34	17		0.84 (0.46-1.54)
Metastasis status			**0.0128[c]**	
No metastasis >5 years	343	215		1.00
Metastasis <5 years	48	14		**0.46 (0.25-0.86)[c]**

[a]Based on two-sided Chi-square test without Yate's correction.

[b]The ORs were estimated with multivariate logistic regression analysis.

[c]Statistically identified as significant.