Short Communication

Polymorphisms of Methylene-tetrahydrofolate Reductase and Risk of Lung Cancer: A Case-Control Study[1]

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Abstract

Previous studies have suggested that low folate intake is associated with increased risk of lung cancer. Methylene-tetrahydrofolate reductase (MTHFR) is one of the enzymes involved in folate metabolism and is thought to influence DNA methylation and nucleotide synthesis. *MTHFR* is highly polymorphic, and the variant genotypes result in decreased MTHFR enzyme activity and lower plasma folate level. Therefore, we hypothesized that these variant genotypes may play a role in the etiology of lung cancer. To test this hypothesis, we investigated the association between two common *MTHFR* polymorphisms (C677T and A1298C) and risk of lung cancer in a non-population-based case-control study of 550 histologically confirmed lung cancer cases and 554 healthy controls. The subjects were non-Hispanic whites, and the controls were frequency-matched to the cases by age (±5 years), sex, and smoking status (ever or never). Folate intake and alcohol consumption were estimated from a self-administered food-frequency questionnaire. The cases consumed significantly less folate (162 μg/day/1000 kcal) than the controls did (172 μg/day/1000 kcal; $P = 0.033$). However, we found no evidence for an association between the *MTHFR* C677T and A1298C polymorphisms and risk of lung cancer in either all of the subjects or the low folate intake subgroup; nor did we find evidence for an interaction between these two *MTHFR* polymorphisms and dietary folate intake or alcohol use. In multivariate logistic regression analysis, the adjusted odds ratios and 95% confidence intervals for *MTHFR* C677T were 1.1 (0.8–1.4) for 677CT *versus* 677CC wild type and 1.1 (0.7–1.7) for 677TT *versus* 677CC, and for *MTHFR* A1298C, they were 1.0 (0.8–1.3) for 1298AC *versus* 1298AA wild type and 1.1 (0.7–1.8) for 1298CC *versus* 1298AA. These results suggest that the *MTHFR* C677T and A1298C polymorphisms by themselves do not play an important role in the etiology of lung cancer.

Introduction

Numerous epidemiological studies (1) have provided evidence that consumption of vegetables and fruit is associated with a reduced risk of cancer, including lung cancer. A number of constituents of vegetables and fruits are thought to contribute to this protective effect. One is folate, often in the form of folic acid (2). Recent results from the Netherlands Cohort Study on Diet and Cancer (3) on the association between vegetable and fruit consumption and cancer incidence revealed that of the nutrients studied, folate exhibited the strongest and most consistent protective effect against lung cancer. A highly significant inverse association between folate intake and lung cancer was also reported in the New York State Cohort Study (4). Two small randomized folate chemoprevention studies have shown that folate supplementation in conjunction with vitamin B_{12} supplementation can reverse purported precursors of squamous cell carcinoma of the lung (5), providing a biological basis for the protective effect of folate in reducing lung cancer risk.

MTHFR[3] is one of the enzymes involved in folate metabolism (6). MTHFR plays a central role in the provision of methyl groups by reducing 5,10-methylene-tetrahydrofolate to 5-methyl-tetrahydrofolate, the dominant circulating form of folate that serves as a substrate for the remethylation of homocysteine to methionine with subsequent production of *S*-adenosylmethionine, the universal donor of methyl, required for DNA methylation (7). DNA methylation has been suggested as one of the molecular mechanisms by which gene expression is regulated (8). For instance, hypomethylation is associated with activation of oncogenes, and promoter hypermethylation is associated with loss of function of tumor suppressor genes (9). A recent study (10) found that individuals homozygous for the C677T polymorphism have undermethylated genomic DNA in their peripheral leukocytes, a characteristic of many types of cancer. MTHFR is also involved in the production of dTMP via thymidylate synthase and purine synthesis. Therefore, MTHFR is also thought to play a role in the provision of nucleotides essential for DNA synthesis and repair (11), and we have demonstrated previously (12) in a subset of this study population that reduced DNA repair of tobacco-induced DNA damage is implicated in the etiology of lung cancer.

MTHFR is highly polymorphic in the general population. Two common polymorphisms, C677T and A1298C, have been identified (13, 14). The nucleotide 677 polymorphism results in an alanine to valine (C $\rightarrow$ T) substitution (13). Individuals with the variant 677TT genotype have about 30% of the *in vitro*

[3] The abbreviations used are: MTHFR, 5,10-methylene-tetrahydrofolate reductase; OR, odds ratio; CI, confidence interval.

MTHFR enzyme activity of those with the 677CC wild-type genotype, whereas heterozygotes (677CT) have about 65% of normal enzyme activity (15). Up to 15% of the population is homozygous 677TT for the variant, which is associated with higher plasma homocysteine levels and reduced plasma folate levels (16). The second common *MTHFR* polymorphism, a glutamate to alanine (A → C) change at position 1298, also influences the specific activity of the enzyme, homocysteine levels, and plasma folate concentration but to a lesser extent than the C677T polymorphism does (14).

Previous studies (17) of colorectal cancer reported a significantly decreased risk of colorectal cancer associated with the 677TT genotype that was not observed among those with low folate intakes or serum levels. Recently, another study (18) reported that individuals with the *MTHFR* 677TT, 1298AC, and 1298CC genotypes have reduced risk of adult acute lymphocytic leukemia. However, the association between these two common *MTHFR* polymorphisms and the risk of lung cancer has not been examined. Because the *MTHFR* polymorphisms reduce enzyme activity and low dietary intake of folate is associated with increased risk of lung cancer, we hypothesized that the *MTHFR* polymorphisms are associated with risk of lung cancer. We tested this hypothesis by genotyping our specimens from a non-population-based case-control study of lung cancer for these two *MTHFR* polymorphisms.

Materials and Methods

Study Subjects. The lung cancer patients and control subjects were selected consecutively from an ongoing case-control study of lung cancer conducted in the Department of Epidemiology at The University of Texas M. D. Anderson Cancer Center. The recruitment of the study subjects has been described in detail elsewhere (12). Briefly, the cases were patients registered from February 1996 to August 2000 at The University of Texas M. D. Anderson Cancer Center with newly diagnosed histologically confirmed primary lung cancer. They had been referred for diagnosis or definitive treatment and had received no previous radiotherapy or chemotherapy. The control subjects were identified from a control-pool database established from registrants of a large managed care system, Kelsey-Seybold Clinic, in the Houston metropolitan area. Control subjects were frequency-matched to the cases by age, sex, and smoking status. Because there were ethnic differences in the distribution of *MTHFR* genotype frequencies and few minority patients were recruited, we selected only non-Hispanic whites for this analysis. Data on demographics, risk factors, and dietary intake of folate were collected by personal interviews using a risk factor questionnaire (12) and a modified version of the Health Habits and History Questionnaire of the National Cancer Institute (19). Blood (20 ml) was drawn into heparinized tubes for DNA extraction and PCR-RFLP analysis. The study was approved by the Institutional Review Boards of The University of Texas M. D. Anderson Cancer Center and the Kelsey Seybold Clinic.

MTHFR **Genotyping.** The leukocyte cell pellet obtained from the 200-μl buffy coat by centrifugation of 1 ml of whole blood was used for DNA extraction. The Qiagen DNA Blood Mini Kit (Qiagen, Inc., Valencia, CA) was used according to the manufacturer's instructions to obtain genomic DNA. The DNA purity and concentration were determined by spectrophotometric measurement of absorbance at 260 and 280 nm.

The *MTHFR* C677T and A1298C polymorphisms were determined by using the primers and PCR-RFLP method described previously (15, 20). The amplified fragments targeted the sites of polymorphisms: the 198-bp fragment for *MTHFR* C677T containing the C → T bp substitution at nucleotide 677 that creates a *Hin*fI restriction site and the 163-bp fragment for *MTHFR* A1298C containing the A → C substitution at nucleotide 1298 that abolishes an *Mbo*II restriction site. The PCRs were performed by using a PTC-200 DNA Engine (Peltier Thermal Cycler; MJ Research Inc., Watertown, MA). Therefore, *Hin*fI and *Mbo*II (New England BioLabs, Beverly, MA) were used to detect the C677T and A1298C polymorphisms, respectively. The digestion products were visualized with ethidium bromide after electrophoresis on 3% NuSieve 3:1 agarose gels (FMC BioProducts, Rockland, ME) for the C677T polymorphism and on 4% Metaphor gels (FMC BioProducts) for the A1298C polymorphism and were photographed with Polaroid film under UV light. 677CC wild-type homozygotes were identified by the presence of only a 198-bp fragment. 677CT heterozygotes were identified by 198-, 175-, and 23-bp fragments, and 677TT homozygotes were identified by 175- and 23-bp fragments. 1298AA wild-type homozygotes produce five fragments of 56, 31, 30, 28, and 18 bp. The 1298AC heterozygotes produce six fragments of 84, 56, 31, 30, 28, and 18 bp, and the 1298CC homozygous variants produce four fragments of 84, 31, 30, and 18 bp. Genotyping was performed on batches of equal numbers of case and control subjects, and all of the laboratory personnel were blinded as to case and control status. About 10% of the samples were randomly genotyped again, and the reproducibility was 100%.

Statistical Analysis. Differences in select demographic variables, smoking, alcohol consumption, and dietary folate intake between cases and controls were evaluated by the χ^2 and Student t tests. The associations between lung cancer and *MTHFR* genotypes were estimated by computing the ORs and their 95% CIs from both univariate and multivariate logistic regression analyses. Stratification analysis was used to study subgroups of age, sex, dietary folate intake, smoking, and alcohol consumption. Logistic regression was also used to assess possible interactions between *MTHFR* genotypes and folate intake and other select risk factors of lung cancer. Those who had smoked less than 100 cigarettes in their lifetimes were defined as never-smokers. Those who had quit smoking for more than 1 year previously were considered former smokers, and the rest were considered current smokers. Those who drank alcoholic beverages less than once a week for the previous years were defined as nondrinkers. Those who had been drinkers but had quit drinking more than 1 year previously were called former drinkers, and the rest were called current drinkers. All of the statistical analyses were performed with Statistical Analysis System software (v.6.12; SAS Institute, Cary, NC).

Results

Select characteristics of the 550 cases and 554 controls are summarized in Table 1. There were more female subjects and current smokers among cases than controls, but these differences were not statistically significant. On average, the cases were 1.7 years older and had smoked longer than the controls. The controls were more likely to be current alcohol drinkers than the cases, although there was no significant difference in average alcohol consumption between cases and controls. The mean folate intake from diet was 317 μg/day in the cases and 331 μg/day in the controls ($P = 0.121$). After adjustment by total calorie intake, the cases had an adjusted folate intake of 162 μg/day/1000 kcal, which was statistically significantly lower than that in the controls (172 μg/day/1000 kcal; $P = 0.033$; Table 1).

Table 1   Characteristics of lung cancer cases and controls

	Cases		Controls		P^a
	No.	(%)	No.	(%)	
All of the subjects	550	(100.0)	554	(100.0)	
Age (yr)					0.100
<55	140	(25.5)	173	(31.2)	
55–64	185	(33.6)	176	(31.8)	
≥65	225	(40.9)	205	(37.0)	
Sex					0.093
Male	283	(51.5)	313	(56.5)	
Female	267	(48.5)	241	(43.5)	
Smoking status[b]					0.163
Nonsmoker	54	(10.5)	48	(9.5)	
Former smoker	235	(45.6)	260	(51.5)	
Current smoker	226	(43.9)	196	(39.0)	
Alcohol status[b]					0.001
Nondrinker	197	(39.7)	129	(25.3)	
Former drinker	148	(29.8)	153	(30.1)	
Current drinker	151	(30.5)	227	(44.6)	
Folate supplement[b]					0.712
Yes	271	(54.6)	284	(55.8)	
No	225	(45.4)	225	(44.2)	
Continuous variables[c]					
Age (yr)	62.1 ± 9.5	(34–87)	60.4 ± 10.4	(19–89)	0.005
Pack-yr	51 ± 36	(0–192)	45 ± 33	(0–189)	0.004
Alcohol (g/day)	8.8 ± 18.6	(0–128)	9.2 ± 16.8	(0–157)	0.721
Calories (kcal/day)	2047 ± 705	(597–5443)	2020 ± 690	(570–4553)	0.534
Folate (μg/day)	317 ± 127	(103–973)	331 ± 137	(92–1233)	0.121
Folate (μg/day/1000 kcal)	162 ± 62	(43–489)	172 ± 69	(65–805)	0.033

[a] Two-sided χ^2 test or t test.
[b] Information on smoking status was available for 515 cases and 504 controls, and information on alcohol and folate intake was available for 496 cases and 509 controls.
[c] Mean ± SD (range).

The *MTHFR* C677T and A1298C allele frequency and genotype distributions in cases and controls are summarized in Table 2. The distributions of the genotypes among the controls were in Hardy-Weinberg equilibrium (χ^2 test, $P = 0.90$ for the C677T polymorphism and $P = 0.43$ for the A1298C polymorphism). We observed identical 677T allele and 677TT genotype frequencies of 0.33 and 0.10, respectively, in the case and control groups. The frequencies of the 1298C allele and 1298CC genotype were 0.30 and 0.07, respectively, in controls, which were also very similar to the values in cases (0.30 and 0.08, respectively). There were no statistically significant differences in genotype frequencies between cases and controls ($P = 0.99$ for C677T and 0.93 for A1298C). No subjects had more than two variant alleles, *i.e.,* individuals homozygous for the variant allele at one site were always homozygous wild type at the other site (677TT/1298AA or 1298CC/677CC), suggesting that having more than two mutations may be embryonically lethal.

On logistic regression analysis, the variant *MTHFR* genotypes 677TT and 677CT were not significantly associated with risk of lung cancer when compared with the 677CC genotype (adjusted OR, 1.1; 95% CI, 0.8–1.4 for 677CT; and adjusted OR, 1.1; 95% CI, 0.7–1.7 for 677TT; Table 3). Similarly, there was no significant association between *MTHFR* A1298C genotype and risk of lung cancer (adjusted OR, 1.0; 95% CI, 0.8–1.3 for 1298AC; and adjusted OR, 1.1; 95% CI, 0.7–1.8 for 1298CC; Table 3). There were also no consistent trends in risk across these two genotypes within different sex and age groups (data not shown).

Associations between the *MTHFR* genotype and lung cancer stratified on dietary folate intake, smoking, and alcohol use were also evaluated (data not shown). Overall, there was no evidence of any association between the *MTHFR* genotype and risk of lung cancer among the different subgroups for either the C677T or the A1298C polymorphism. There was a weak trend toward an association between the variant allele and lung cancer risk among those with medium folate intake (for 677CT and 677TT) and those with low folate intake (for 1298AC and 1298CC); however, the CIs for these subgroups were wide. There was no evidence of an interactive effect on lung cancer risk between low folate intake and *MTHFR* genotypes. There was also no consistent trend in association between *MTHFR* genotypes and lung cancer within different subgroups of smoking and alcohol consumption, although an elevated risk was evident for former drinkers with the 677TT genotype, but this could be attributable to chance.

Discussion

The identification of common polymorphisms in the *MTHFR* gene and the demonstration that these C677T and A1298C variant genotypes are correlated with *in vitro* MTHFR activity and blood folate levels have attracted considerable research interest in recent years. However, there have been only a few studies of the association between the *MTHFR* C677T polymorphism and risk of cancer, mostly on colorectal cancer (17, 18, 21, 22). Colorectal cancer may be of interest because colorectal carcinogenesis is facilitated by the high rate of proliferation of the epithelium, and highly proliferative tissues tend to have a high demand for folate that is required for methylation, nucleotide synthesis, and DNA repair.

To the best of our knowledge, this is the first report to examine the association between the *MTHFR* gene polymorphisms and risk of lung cancer. Because there were more

Table 2 *MTHFR* C677T and A1298C genotype distribution and allele frequencies in lung cancer patients and healthy controls

		C677T (Ala → Val)			Allele frequency	
		CC (%)	CT (%)	TT (%)	C	T
Cases ($n = 550$)						
A1298C (Glu → Ala)					0.67	0.33
AA (%)		76 (14)	128 (23)	57 (10)		
AC (%)		122 (22)	124 (23)			
CC (%)		43 (8)				
Allele frequency						
A	0.70					
C	0.30					
Controls ($n = 554^a$)						
A1298C (Glu → Ala)					0.67	0.33
AA (%)		76 (14)	132 (24)	57 (10)		
AC (%)		129 (23)	120 (22)			
CC (%)		40 (7)				
Allele frequency						
A	0.70					
C	0.30					

[a] All the observed genotype frequencies for the C677T and A1298C polymorphisms in the controls were not statistically different from those ($P = 0.900$ and $P = 0.430$, respectively) calculated on the basis of Hardy-Weinberg's equation ($p^2 + 2pq + q^2$).

Table 3 Associations between *MTHFR* genotypes and lung cancer

Genotype	Cases ($n = 550$)	Controls ($n = 554$)	OR^a (95% CI)
C677T[b]			
CC	241 (43.8)	245 (44.2)	1.0
CT	252 (45.8)	252 (45.5)	1.1 (0.8–1.4)
TT	57 (10.4)	57 (10.3)	1.1 (0.7–1.7)
A1298C[c]			
AA	261 (47.5)	265 (47.8)	1.0
AC	246 (44.7)	249 (45.0)	1.0 (0.8–1.3)
CC	43 (7.8)	40 (7.2)	1.1 (0.7–1.8)

[a] ORs were adjusted for age, sex, smoking status, alcohol use, and calorie-adjusted folate intake in a logistic regression model.
[b] There was no difference in the C677T genotype distributions between cases and controls ($P = 0.991$).
[c] There was no difference in the A1298C genotype distributions between cases and controls ($P = 0.931$).

alcohol users among the controls, this may lead to underestimation of the risk associated with low intake of folate and the polymorphisms studied, because alcohol may reduce the bioavailability of folate *in vivo* (17). However, we have included a large number of subjects in this study but found no evidence of an association between the *MTHFR* C677T and A1298C polymorphisms and risk of lung cancer in the multivariate analysis. Nor did we observe a significant interaction between the *MTHFR* polymorphisms and dietary folate intake. Therefore, the data do not support the hypothesis that *MTHFR* C677T and A1298C genotypes are associated with risk of lung cancer.

Early reports suggested that the *MTHFR* 677TT genotype appeared to protect against colorectal cancer (17) and acute lymphocytic leukemia (18). One study reported an approximately 50% reduction in colorectal cancer risk in both men and women with the 677TT genotype compared with persons with the 677CC wild-type genotype (17). This protection was not evident for those who had low blood folate levels or low dietary folate intake. However, more recent studies (23, 24) suggested that the 677TT genotype was a risk factor for colorectal adenoma in subjects who reported low dietary folate intake, although the overall frequencies of the 677TT genotype were very similar in colorectal adenoma cases and controls (10 *versus* 11%, respectively). There was a lack of association between the 677TT genotype and colorectal hyperplastic polyps (21) and colorectal adenomas (25). Other studies have implicated the 677TT genotype in increased risk of cervical intraepithelial neoplasia (26) and endometrial cancer (27).

However, few studies have investigated these two polymorphisms simultaneously. Only one evaluated the *MTHFR* A1298C polymorphism and risk of cancer (18). Individuals with the *MTHFR* 677TT, 1298AC, and 1298CC genotypes were found to have a decreased risk of adult acute lymphocytic leukemia but not acute myeloid leukemia. The results suggested that folate inadequacy may play a key role in the development of acute lymphocytic leukemia.

In our study reported here, the frequencies of the variant *MTHFR* 677TT genotype and 677T allele were 0.10 and 0.33,

respectively, and those of the *MTHFR* 1298CC genotype and 1298C allele were 0.07 and 0.30, respectively, which were consistent with those reported for the control subjects in previous studies in the United States. Skibola *et al.* (18) reported genotype and allele frequencies of 0.12 and 0.29 for the *MTHFR* 677TT genotype and 677T allele, respectively, and 0.11 and 0.33 for the *MTHFR* 1298CC genotype and 1298C allele, respectively, in 369 Caucasian control subjects. Recently, Ulrich *et al.* (21, 23) reported genotype and allele frequencies of 0.11 and 0.32 for the *MTHFR* 677TT genotype and 677T allele, respectively, in 645 control subjects (97% were whites). In addition, we observed that individuals homozygous for the mutation at one site were always homozygous wild type at the other site, which validated the results of disequilibrium between these two polymorphisms from a report (28) with a relatively small sample size of Caucasians.

We noted that the mean calorie-adjusted folate intake from diet was statistically significantly lower in lung cancer cases than in controls. Although the estimates of dietary folate intake were derived from questionnaire data that may not represent the actual amount of intake, the lower mean folate intake from diet in the cases than in the controls is consistent with a protective effect of folate. The lack of association between the *MTHFR* C677T and A1298C genotypes and risk of lung cancer suggests that the *MTHFR* variant genotype did not have an effect on lung cancer or that other molecular mechanisms such as DNA damage and repair may play a major role in the etiology of lung cancer in this study population (12).

In conclusion, our study does not provide evidence for associations between the *MTHFR* C677T and A1298C variant genotypes and risk of lung cancer, with and without dietary folate intake, in this study population. Because of the design of the study and crude measurement for dietary folate intake, these results may not be generalizable to the general population. A large prospective study is needed to verify our findings. However, we cannot rule out the possibility that other as yet unidentified alterations in genes involved in folate metabolism, DNA methylation, and DNA repair influence the risk of developing lung cancer. Indeed, suboptimal DNA repair capacity is associated with increased risk of lung cancer (12). It is likely that insufficient dietary folate intake may lead to lung cancer by influencing the DNA repair pathway. We are currently testing this hypothesis and investigating the association between dietary folate intake and DNA repair capacity and their combined effect on risk of lung cancer.

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