Relationship between Methylenetetrahydrofolate Reductase *C677T* and *A1298C* Genotypes and Haplotypes and Prostate Cancer Risk and Aggressiveness

Mine S. Cicek,[1] Nora L. Nock,[2] Li Li,[2] David V. Conti,[3] Graham Casey,[1] and John S. Witte[4]

[1]Department of Cancer Biology, Cleveland Clinic Lerner College of Medicine, Cleveland, Ohio; [2]Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, Ohio; [3]Department of Preventive Medicine, University of Southern California, Los Angeles, California; and [4]Department of Epidemiology and Biostatistics, University of California-San Francisco, San Francisco, California

Abstract

Previous reports indicate that polymorphisms in the *MTHFR* gene play a role in cancer development, but their potential impact on prostate cancer has not been well studied. Here, we evaluate the association between two *MTHFR* polymorphisms, *C677T* and *A1298C*, and prostate cancer risk and aggressiveness in a moderately large family-based case-control study (439 cases and 479 sibling controls). Among all study subjects, we observed no association between the *C677T* variant and prostate cancer but a slight positive association between the *A1298C* variant and risk of this disease [odds ratio (OR) 1.41, 95% confidence interval (CI) 0.96-2.06; *P* = 0.08]. When stratifying the study population by disease aggressiveness at diagnosis, the *C677T* variant was positively associated with risk among men with less advanced disease (OR 1.86, 95% CI 1.00-3.46; *P* = 0.05). In contrast, when looking at men with more advanced disease, the *C677T* variant was inversely associated with risk (OR 0.51, 95% CI 0.32-0.82; *P* = 0.01), whereas the *A1298C* variant was positively associated with risk (OR 1.79, 95% CI 1.06-3.02; *P* = 0.03). Furthermore, the *677T-1298A* haplotype was positively associated with prostate cancer among men with less advanced disease (OR 1.84, 95% CI 1.07-3.16; *P* = 0.03) and inversely associated with risk of more advanced disease (OR 0.47, 95% CI 0.29-0.76; *P* = 0.002). Our findings suggest that *677T* and *1298A*, or another variant on their haplotype, may be associated with a reduced risk of progression to more advanced prostate cancer. (Cancer Epidemiol Biomarkers Prev 2004;13(8):1331–6)

Introduction

Methylenetetrahydrofolate reductase (MTHFR) plays a key role in the metabolism of folates, which are important nutrients required for both DNA synthesis and DNA methylation. In particular, MTHFR irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulating folate and the carbon donor for remethylation processes (1-3). This conversion is critical in controlling intracellular homocysteine levels and maintaining adequate levels of *S*-adenosylmethionine, the universal methyl donor for all protein, lipid, and DNA methylation reactions (4-6). A reduction in 5-methyltetrahydrofolate levels would lower the source of methyl groups for DNA methylation, an important epigenetic process that plays a critical role in gene regulation (7, 8). Conversely, a reduction in the levels of the MTHFR substrate 5,10-methylenetetrahydrofolate would result in decreased thymidylate biosynthesis, leading to deoxynucleotide pool imbalances and increased uracil misincorporation into DNA, potentially causing double-strand breakage (9).

Two common polymorphisms in the *MTHFR* gene, *C677T* and *A1298C*, have been described (10-12). The *C677T* variant lies in exon 4 at the folate binding site of the *MTHFR* gene and results in the substitution of an alanine by a valine (Ala^{222}Val) residue (10, 13). This substitution causes reduced activity and thermolability of MTHFR and results in lower levels of 5-methyltetrahydrofolate, an accumulation of 5,10-methylenetetrahydrofolate and increased plasma homocysteine levels (13-17). The *A1298C* variant (Glu^{429}Ala) lies in exon 7 and results in a decrease in MTHFR enzymatic activity that is more pronounced in homozygotes (*C/C*) than heterozygotes (*A/C*), although it does not result in a thermolabile protein (18). Furthermore, unlike the *MTHFR C677T* variant, the *A1298C* variant allele is not associated with lower folate plasma level (19). However, compound heterozygotes for the *A1298C* and *C677T* genotypes tend to have increased plasma homocysteine levels and decreased plasma folate levels similar to *C677T* homozygotes (12).

Several studies have reported that the *MTHFR C677T* variant is inversely associated with the risk of colorectal carcinoma, primarily among those with a high methyl diet or low alcohol consumption (20-26). Inverse associations were also observed between this variant and acute lymphocytic leukemia (27), childhood leukemia (28), and

malignant lymphoma (29). However, no association was found between the *C677T* variant and colon adenomas (22), hyperplastic polyps (30), acute myeloid leukemia (27), lung cancer (31), or transitional cell carcinoma of the urinary bladder (32). In contrast, a positive association was reported between the *C677T* variant and cancers of the endometrium (33), esophagus (34), cervix (35), and cervical dysplasia (36).

Studies investigating the *MTHFR A1298C* variant have found inverse associations with colorectal cancer (24), breast cancer (37), acute lymphocytic leukemia (27), and childhood leukemia (28). However, a positive association was reported between the *A1298C* variant and esophageal cancer (34), although studies of acute myeloblastic leukemia (27) and lung cancer (31) were equivocal.

Whereas published studies suggest that the *MTHFR C677T* and *A1298C* variants may affect certain cancers, knowledge about their potential impact on prostate cancer is limited. Two studies have looked at the *C677T* variant and prostate cancer: one reported a positive association overall (38), whereas another found a weak positive association with higher tumor grade (39). Here, we give results from a sibling-based case-control study evaluating the relationship between both *C677T* and *A1298C* variants and prostate cancer risk and aggressiveness.

Materials and Methods

Subjects. The study design and population have been described elsewhere (40). Briefly, a set of siblings (n = 918; 439 cases and 479 controls from 413 families) was recruited from the major medical institutions in the greater Cleveland, OH area and from the Henry Ford Health System (Detroit, MI). Note that, in comparison with our previous publications (e.g., ref. 40), there is one fewer case and control included here. This was due to one control being diagnosed with prostate cancer after initially enrolling in our study; this individual and his case sibling have been removed from the current analysis. Institutional review board approval was obtained from the participating institutions, and all study participants gave informed consent.

Sibling sets consisted of men with prostate cancer diagnosed at age ≤73 years and at least one brother without prostate cancer. All controls were <8 years younger than their brother's age at diagnosis. These age restrictions were used in an attempt to increase the potential for genetic factors affecting disease and to reduce the probability that the controls were not unaffected due simply to being of a younger age. The disease status of unaffected brothers was further confirmed through testing of prostate-specific antigen levels whenever possible (93% of controls). Participants with prostate-specific antigen levels >4 ng/mL were informed and advised to investigate their disease status with their physician. They were retained in the study as controls, unless a subsequent diagnosis of prostate cancer was made, at which time they were reclassified as cases. Keeping them in the study is important, because automatically excluding men with elevated prostate-specific antigen levels regardless of their ultimate prostate cancer status can lead to biased estimates of

association (41, 42). Furthermore, by using a sibling-based study design, we are assured that our controls have been ascertained from the case's genetic source population, excluding the potential for bias due to population stratification (43).

Most of the 413 sibships had one case and one or more controls; 18 families had two cases, and four families had three or more cases (and at lease at one control). Hence, families with multiple affected men are unlikely to affect our results, especially because we are looking at common polymorphisms with potentially low impact on prostate cancer susceptibility (44).

Eight hundred and thirty-two of the brothers are Caucasians (91%), 76 are African American (8%), 8 are Latinos (1%), and 2 are Asian American. The case's clinical characteristics at diagnosis (e.g., Gleason score and tumor stage) were obtained from medical records, and their disease status was confirmed histologically. All study subjects completed a self-administered health and habits questionnaire, a food frequency questionnaire (Channing Laboratory, Boston, MA), and donated a blood sample.

Genotyping. Standard venipuncture was used to collect blood samples from all study participants in tubes with EDTA as an anticoagulant. Genomic DNA was extracted from buffy coats using the QIAmp DNA Blood Kit (Qiagen, Inc., Valencia, CA). All purified DNA samples were diluted to a constant DNA concentration of 10 ng/μL in 10 mmol/L Tris-5 mmol/L EDTA buffer (pH 8). The genotyping protocols for the detection of the *MTHFR C677T* and *A1298C* polymorphisms were adapted from Frosst et al. (13) and Weisberg et al. (12), respectively.

We detected the presence of the *MTHFR C677T* polymorphism by amplifying genomic DNA with the forward primer 5′-GGTCAGAAGCATATCAGTCAT-GAG-3′ and the reverse primer 5′-CTGGGAAGAACT-CAGCGAACTCAG-3′. The PCR amplification parameters were a 2-minute initial denaturation cycle at 94°C and 30 cycles each of 30 seconds at 94°C, 30 seconds at 62°C, and 30 seconds at 72°C followed by a 7-minute final elongation cycle at 72°C. The 494-bp PCR product was digested with *Hin*fI (New England Biolabs, Beverly, MA) at 37°C for 3 hours. Digested products were separated by electrophoresis on a 1.5% agarose gel. Wild-type alleles (*677C*) resulted in 100- and 394-bp fragments following restriction enzyme digestion. The variant alleles (*677T*) resulted in 100-, 165-, and 229-bp products after digestion.

We identified the *MTHFR A1298C* variant by amplifying genomic DNA with the forward primer 5′-AAGGAGGAGCTGCTGAAGATG-3′ and the reverse primer 5′-CTTTGCCATGTCCACAGCATG-3′. The PCR amplification parameters were a 5-minute initial denaturation cycle at 95°C and 30 cycles each of 30 seconds at 94°C, 30 seconds at 63°C, and 30 seconds at 72°C followed by a 10-minute final elongation cycle at 72°C. The 237-bp PCR product was digested with *Mbo*II (New England Biolabs) at 37°C for 1 hour. Digested products were separated by electrophoresis on a 6% acrylamide gel. Wild-type alleles (*1298A*) resulted in 27-, 28-, and 182-bp fragments, and variant alleles (*1298C*) resulted in 127- and 210-bp fragments following restriction enzyme digestion.

To ensure quality control of both sets of genotyping results, 5% of samples were randomly selected and genotyped by a second investigator and 1% was sequenced using a 377 ABI automated sequencer (all results were concordant).

Statistical Analysis. We first calculated *MTHFR* genotype frequencies and tested for Hardy-Weinberg equilibrium within the major ethnic groups and stratified by case-control status. Next, we estimated haplotypes with the program PHASE (45; again within major ethnic groups stratified by case-control status) and calculated the linkage disequilibrium between *C677T* and *A1298C* alleles. Finally, conditional logistic regression—with family as the matching variable and a robust variance estimator that incorporates familial correlations—was used to estimate odds ratios (OR) and 95% confidence intervals (CI) for the association among genotypes, haplotypes, and prostate cancer. In addition to an independent analysis of genotypes comparing one or more variants with the nonvariant, both genes were simultaneously included in the same regression model to assess the potential impact of the *C677T* and *A1298C* alleles on prostate cancer.

To investigate the potential effect of the genetic variants on disease aggressiveness, we stratified the analyses by the cases' clinical characteristics at diagnosis. Aggressiveness was defined as "low" if a case's Gleason score was <7 and their tumor category was <T2c and "high" if their Gleason score was ≥7 and their tumor category was ≥T2c. The tumor category reflects the TNM system (46). The regression models adjusted for potential confounding by age, folate intake, calories, alcohol consumption, smoking, and body mass index (all continuous). All *P* values are from two-sided tests, and analyses were undertaken with S+ software (version 6.0, Insightful Corp., Seattle, WA).

Results

The mean (SD) age at diagnosis among cases was 61.0 (6.7) years, and the mean (SD) age at enrollment for controls was 62.7 (9.1) years (Table 1). The interquartile range in these ages was 56 to 66 years for cases and 56 to 69 years for controls.

The *C677T* and *A1298C* alleles were in Hardy-Weinberg equilibrium within ethnic groups (*P* > 0.3), and the genotype frequencies differed slightly between cases and controls (Table 1). The *MTHFR* alleles were found to be in strong linkage disequilibrium (*D′* ≥ 0.99), and one of the four possible haplotypes (*677T-1298C*) was only observed in a single brother pair (and hence is not included in our regression analysis).

Table 2 provides the matched OR for the association between *MTHFR* genotypes and prostate cancer. Among all study subjects, there was no association between the *MTHFR C677T* variant genotypes and prostate cancer (Table 2). For the *MTHFR A1298C* variant, we observed a weak positive association with prostate cancer. Comparing men with at least one *1298C* allele with individuals homozygous for the *1298A* allele gave an OR (95% CI) of 1.41 (0.96-2.06; *P* = 0.08). The estimated *MTHFR C677T* and *A1298C* haplotypes were not associated with prostate cancer among all study subjects (Table 2).

Table 1. Characteristics of the family-based case-control study population including *MTHFR* genotype and haplotype counts

	Cases (*n* = 439)	Controls (*n* = 479)
Age (y), mean (SD)	61.0 (6.7)	62.7 (9.1)
Ethnicity, *n* (%)		
Caucasian	397 (90)	437 (91)
African American	38 (9)	38 (8)
Other	5 (1)	5 (1)
Genotypes, *n* (%)		
677 CC	214 (49)	219 (46)
677 CT	182 (41)	199 (41)
677 TT	43 (10)	61 (13)
1298 AA	195 (44)	233 (49)
1298 AC	205 (47)	201 (42)
1298 CC	39 (9)	44 (9)
Haplotypes, *n* (%)*		
677C-1298A	330 (38)	349 (36)
677C-1298C	280 (32)	288 (30)
677T-1298A	267 (30)	320 (33)
677T-1298C	1 (0.1)	1 (0.1)

*Counts reflect the number of chromosomes.

When stratifying by disease aggressiveness, we observed strong effect modification (Table 3). In particular, among men with low aggressive disease at diagnosis, the *C677T* variant was positively associated with prostate cancer (OR 1.86, 95% CI 1.00-3.46; *P* = 0.05), whereas, in men with high aggressive disease, the variant was inversely associated with disease (OR 0.51, 95% CI 0.32-0.82; *P* = 0.01). There was a slight trend across genotypes, as each additional copy of the *677T* variant seemed to increase (or decrease) the respective ORs (Table 3). In contrast, the *A1298C* variant was positively associated with disease among men with more advanced prostate cancer (OR 1.79, 95% CI 1.06-3.02; *P* = 0.03). Including both polymorphisms in a single model slightly weakened these individual associations but did not materially alter the OR estimates (data not shown).

When looking at the *MTHFR C677T* and *A1298C* haplotypes, as expected based on the genotype-level results, there was effect modification across prostate cancer aggressiveness at diagnosis (Table 3). Specifically, within the low aggressiveness stratum, comparing the *677T-1298A* haplotype with the *677C-1298A* haplotype gave an OR (95% CI) of 1.84 (1.07-3.16; *P* = 0.03). In addition, within the high aggressiveness stratum, this haplotype was inversely associated with prostate cancer (OR 0.47, 95% CI 0.29-0.76; *P* = 0.002).

Restricting our analyses to Caucasians only or to matched sets where the control was at most 5 years younger than their corresponding sibling's (i.e., case's) age at diagnosis did not materially alter our results (data not shown).

Discussion

In our entire sample of prostate cancer cases and their brother controls, we observed no association for the *MTHFR C677T* variant and a weak positive association for the *A1298C* variant. The latter association was strengthened when restricting our analyses to men with more advanced prostate cancer at diagnosis. Moreover,

Table 2. Association between *MTHFR C677T* and *A1298C* genotypes/haplotypes and prostate cancer risk

MTHFR Variant	Cases/Controls	Crude*		Adjusted†	
		OR (95% CI)	P	OR (95% CI)	P
C677T genotype					
CC (reference)	214/219	1.0 (reference)		1.0	
CT	182/199	0.96 (0.68-1.37)	0.83	0.90 (0.63-1.29)	0.57
TT	43/61	0.70 (0.36-1.36)	0.29	0.67 (0.34-1.32)	0.24
CT or TT		0.94 (0.66-1.33)	0.73	0.88 (0.62-1.26)	0.49
A1298C genotype					
AA (reference)	195/233	1.0		1.0	
AC	205/201	1.44 (0.99-2.09)	0.05	1.42 (0.97-2.09)	0.07
CC	39/44	1.04 (0.52-2.11)	0.91	1.07 (0.52-2.18)	0.86
AC or CC		1.42 (0.98-2.05)	0.06	1.41 (0.96-2.06)	0.08
Haplotypes‡					
677C-1298A (reference)	330/349	1.0		1.0	
677C-1298C	280/288	1.17 (0.81-1.67)	0.41	1.12 (0.77-1.63)	0.54
677T-1298A	267/320	0.95 (0.68-1.34)	0.78	0.90 (0.63-1.27)	0.54

*Crude: regression model includes age (continuous).
†Adjusted for age, folate intake, calories, alcohol consumption, pack-years smoking, and body mass index (all continuous).
‡Counts reflect the number of chromosomes. There is one case and one control with the *677T-1298C* haplotype.

in this subset of more aggressive cases, the *C677T* variant and the *677T-1298A* haplotype were inversely associated with prostate cancer. In contrast, among men with less advanced disease, the *C677T* variant and the *677T-1298A* haplotype were positively associated with risk. Our observations suggest that these *MTHFR* variants, or another variant on their haplotype (i.e., in linkage disequilibrium with them), may be involved with the development and progression of prostate cancer.

The results for the *C677T* variant within the aggressiveness stratum are consistent with some but not all published studies, which have reported inverse associations with colorectal cancer (20-26, 47, 48), acute lymphocytic leukemia (27), childhood leukemia (28, 49), and malignant lymphoma (29). Of the two studies to date that have evaluated this variant and prostate cancer, one detected a positive association (38), whereas the second found a weak positive association with higher tumor

grade (39). The first study (38) is consistent with our finding for men with less advanced disease, whereas the second is not (39). In the former study, a positive association was found between the *C677T* variant and several cancers including those of the prostate, colorectum, kidney, and bladder (38). The latter study was relatively small (132 cases and 150 controls), and 84 of the controls were female and hence not really representative of the cases source population because they could never develop prostate cancer (39).

The strong linkage disequilibrium that underlies the *677T-1298A* haplotype has been reported previously (24, 50, 51). In our study, both the *677T-1298A* haplotype, where the variants are in *trans* configuration, and the *C677T* variant showed similar inverse associations for men with more aggressive disease, implying that this effect is driven primarily by the *677T* variant or another variant on the same haplotype. Previous studies have

Table 3. Aggressiveness-stratified ORs for relation between *MTHFR C677T* and *A1298C* genotypes/haplotypes and prostate cancer

MTHFR Variant	Low aggressive*			High aggressive†		
	Cases/Controls	OR (95% CI)‡	P	Cases/Controls	OR (95% CI)‡	P
C677T genotype						
CC (reference)	87/106	1.0 (reference)		119/102	1.0	
CT	77/76	1.85 (0.99-3.46)	0.05	89/104	0.55 (0.34-0.88)	0.01
TT	24/25	2.01 (0.69-5.85)	0.20	17/34	0.22 (0.08-0.61)	0.004
CT or TT		1.86 (1.00-3.46)	0.05		0.51 (0.32-0.82)	0.01
A1298C genotype						
AA (reference)	89/103	1.0		93/118	1.0	
AC	81/83	1.34 (0.71-2.54)	0.36	113/98	1.81 (1.07-3.05)	0.03
CC	18/21	1.00 (0.35-2.86)	1.00	19/23	1.01 (0.36-2.88)	0.98
AC or CC		1.32 (0.70-2.50)	0.39		1.79 (1.06-3.02)	0.03
Haplotypes§						
677C-1298A (reference)	135/164	1.0		176/162	1.0	
677C-1298C	116/124	1.49 (0.85-2.61)	0.17	149/144	0.87 (0.50-1.49)	0.61
677T-1298A	124/125	1.84 (1.07-3.16)	0.03	123/172	0.47 (0.29-0.76)	0.002

*Includes cases with Gleason score <7 and/or tumor stage <T2C and their brothers.
†Includes cases with Gleason score ≥7 and/or tumor stage ≥T2C and their brothers.
‡Adjusted for age, folate intake, calories, alcohol consumption, pack-years smoking, and body mass index (all continuous).
§Counts reflect haplotypes. There is one case and one control with the 677T-1298C haplotype for Low Aggressive.

suggested that the *A1298C* variant may not be an independent risk factor in colorectal cancer (24). However, our data suggest that it may have some function as we found a (weak) positive association between the *1298C* variant and prostate cancer risk.

Significant advances have been made in the past few years in establishing the biological rationale for the association between *MTHFR* variants and cancer (9, 52). Folate derivatives are essential for DNA synthesis and methylation and have a critical role in regulation of gene expression influencing the cancer risk (53-55). Epidemiologic evidence supports a potentially protective role of dietary folate on colon and breast cancer; to our knowledge, only one article has looked at the potential relation of serum folate with prostate cancer (56). This was a case-control study (232 cases and 464 controls) nested within the Finnish α-Tocopherol β-Carotene Cancer Prevention Study of smokers. None of the serum measures were associated with prostate cancer, although weak positive associations were observed for the interaction between alcohol intake and low folate or high homocysteine (56). However, many of the study subjects had inadequate serum vitamin levels (all older male smokers), and no *MTHFR* genotypes were evaluated.

Our observation of an inverse association for the *C677T* variant but a positive association for the *A1298C* variant among men with more advanced disease was unanticipated. Whereas these variants do not result in opposite effects *in vitro*, the functional consequences of the *C677T* variant are much better known than for the *A1298C* variant. The different effects of the variants on prostate cancer risk may be a consequence of their location within the protein and subsequent effect on function. Specifically, the profound impact of the alanine to valine (*C677T*) substitution, compared with glutamate to alanine (*A1298C*), on protein stability and activity could be due to their distinct locations (57, 58). The effect of enzyme activity with the *C677T* polymorphism may be a consequence of being within the region encoding the NH_2-terminal catalytic domain. However, the *A1298C* variant may affect enzyme regulation by *S*-adenosylmethionine, because it is in the COOH-terminal *S*-adenosylmethionine regulatory domain of the enzyme where the MTHFR inhibitor can bind.

There are several explanations for the protective effect of the *677T-1298A* haplotype observed among men with more aggressive disease, one of which is that cells with the haplotype have increased 5,10-methylenetetrahydrofolate levels for DNA synthesis in those individuals with sufficient folate intake (21). A second theory is that the variant *T* allele may be preferentially lost in cancer patients. Loss of heterozygosity at the *MTHFR* locus was observed in 16% to 18% of colorectal tumors with exclusive loss of the variant *T* allele (i.e., 8 in 11 tumors; refs. 59, 60). Up to 59% of ovarian tumors have been reported to show loss of heterozygosity at the *MTHFR* locus, and this was associated with a decrease in MTHFR activity (61). The *MTHFR* allele loss in cases in these studies may suggest that functional MTHFR activity within the tumor may play a role in the survival and progression of disease. However, little is known about the prostate tissue-specific expression and transcriptional regulation of the *MTHFR* gene.

The following limitations to this study merit consideration. By using a family-based design, cases and controls can be overmatched with regard to unmeasured genetic and environmental factors, resulting in reduced power to detect association. Nevertheless, this matching can help control for potential confounding and eliminates the potential for biased results due to population stratification. Another limitation is the recruitment of cases through major medical institutions in Cleveland and Detroit. Whereas these institutions see many of the prostate cancer cases occurring within these geographic regions, the study is not formally population based.

In conclusion, our relatively large family-based case-control study exhibited an inverse association between the *677T* and *1298A* haplotypes and prostate cancer among men with more advanced disease and a positive association among men with less advanced disease. The magnitude of the effects observed here suggests that these alleles, or another variant on their haplotype, provide ~50% change in risk of prostate cancer. These results suggest that folate metabolism may play an important role in prostate cancer development and progression. The association between *MTHFR* variants and prostate cancer may be modulated by other potential enzyme variants involved in folate metabolism as well as other dietary factors (e.g., alcohol and methionine).

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Mine S. Cicek, Nora L. Nock, Li Li, et al.

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