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A Common Variant of the Methylenetetrahydrofolate Reductase Gene (1p36) Is Associated with an Increased Risk of Cancer[1]

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ABSTRACT

Folate metabolism is thought to play an important role in carcinogenesis through its involvement in both DNA methylation and nucleotide synthesis. A common Ala[222]/Val variant in the *methylenetetrahydrofolate reductase* (*MTHFR*) gene leads to a disturbed folate metabolism and is associated with decreased genomic DNA methylation. We previously reported that the *MTHFR* Val/Val genotype was associated with increased cancer mortality in men from a population-based cohort of subjects ages ≥ 85 years. To further explore the deleterious effects of the *MTHFR* genotype, we studied the association of the genotype with cancer risk in 860 men ages 65–84 years who were followed >10 years (Zutphen Elderly Study).

During follow-up, 149 new cases of cancer occurred among the 793 men without cancer at baseline. The risk of developing cancer was 1.80-fold (95% confidence interval, 1.09–3.00) higher among men with the Val/Val genotype than among men with the Ala/Ala genotype. Except for lung cancer [relative risk (RR), 1.15], the risks of common forms of cancers were significantly increased among men with the Val/Val genotype [cancer of the prostate (RR, 3.48); the colorectum (RR, 3.65); the kidney and bladder (RR, 5.48)]. The risks of cancer were particularly increased among men with a lower folate and a higher alcohol intake and men of an older age. In conclusion, our current and previous studies in two independent populations indicate that a common Ala/Val variant in the *MTHFR* gene is a risk factor for cancer in elderly men from the general population. The mechanism underlying this association might involve genomic instability as a result of insufficient methylation of genomic DNA.

INTRODUCTION

Epidemiological studies implicated low folate status in the development of cancer in several organs, including the cervix, colorectum, lung, brain, pancreas, and breast (1). These observations may be explained by the crucial role of folate as the donor of one-carbon groups in both DNA methylation and nucleotide synthesis. In humans, folate deficiency induces decreased DNA methylation (2), which is a nearly universal feature of early tumorigenesis (1). Insufficient methylation of DNA may promote carcinogenesis by the derepression of proto-oncogenes (3, 4) or by the induction of genomic instability (5). Folate is additionally required for the conversion of the nucleotide dUMP to dTMP. An imbalanced nucleotide pool caused by folate deficiency is associated with an increased occurrence of chromosome breaks as a result of the simultaneous removal and repair of adjacent misincorporated uracil bases on opposing DNA strands (6, 7) and may thereby contribute to cancer risk (8).

MTHFR[3] is a key enzyme in folate metabolism and converts 5,10-methylenetetrahydrofolate to 5-methylTHF (Fig. 1). The latter form of folate is used for the remethylation of homocysteine to methionine. DNA methylation is dependent on the synthesis of methionine because its activated form, *S*-adenosyl-methionine, is the methyl donor in this reaction. If not reduced to 5-methylTHF by MTHFR, 5,10-methylenetetrahydrofolate can transfer its methylene group to dUMP to synthesize dTMP or may contribute to purine synthesis. A common alanine 222-to-valine (Ala/Val) variant of the *MTHFR* gene was found to decrease the activity of the enzyme by 70% in homozygotes for the Val-allele (Ref. 9; ~10% of the general population) and leads to a shift in the distribution of different forms of THF at the expense of 5-methylTHF (10). As is consistent with a diminished availability of 5-methylTHF, the Val/Val genotype is associated with elevated plasma homocysteine levels (11) and decreased genomic DNA methylation (12). As yet there is, however, no evidence for a higher resistance to uracil misincorporation associated with the Val/Val genotype (13).

In a previous study, we presented evidence that the Val/Val genotype was associated with a higher mortality rate in men but not women ages ≥ 85 years and that this observation could be attributed to an increased risk of cancer (14). Later, the Val/Val genotype was also found to be associated with an increased prevalence of gastric cancer (15), colorectal cancer with microsatellite instability (16), and cervical dysplasia (17). In contrast, the Val/Val genotype was observed to reduce the risk of colorectal cancer (18, 19) and acute lymphocytic leukemia (20, 21) in other studies. It thus remains unclear which of the putative effects of the genotype, either the deleterious influence on methylation or the advantageous influence on nucleotide synthesis, prevails in determining cancer risk in the general population. Therefore, we examined the association of the *MTHFR* genotype with the risk of cancer in a population-based prospective study among elderly men (the Zutphen Elderly Study; Ref. 22).

MATERIALS AND METHODS

Subjects. The Zutphen Elderly Study is a population-based, longitudinal investigation of risk factors for chronic diseases in elderly men (22). It is an extension of the Dutch contribution to the Seven Countries Study. In 1985, the 555 survivors of the original cohort of 878 and a random sample of 711 men of the same age (65–84 years) also living in Zutphen were approached. Of those invited 74% (939 of 1266), entered the study: 62 had moved or could not be reached; 109 could not be examined because of serious illness; and 156 refused. Complete information on genotype and standard risk factors was available for 860 men and for 804 men, information on diet was also available. The study was approved by the Medical Ethics Committee of Leiden University and informed consent was obtained from all participants.

Baseline Examinations. Baseline medical and diet examinations were carried out between March and June 1985 (22). Information on smoking status and the prevalence of cancer was obtained by a standardized medical ques-

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[3] The abbreviations used are: MTHFR, methylenetetrahydrofolate reductase; 5-methylTHF, 5-methyltetrahydrofolate; CI, confidence interval; RR, relative risk; THF, tetrahydrofolate.

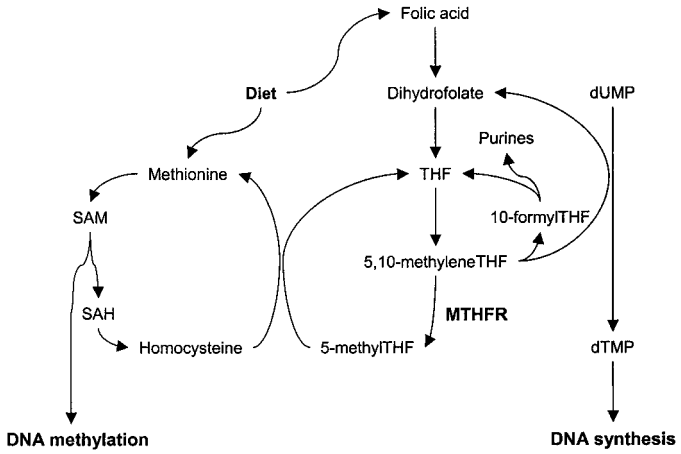

Fig. 1. Competing pathways in folate metabolism. THF, tetrahydrofolate; SAM, *S*-adenosyl-methionine; SAH, *S*-adenosyl-homocysteine.

tionnaire. Data on baseline prevalence of all cancers were verified with hospital discharge data and written information from the general practitioner. Usual food intake of the participants in the month before the interview was recorded by trained dieticians who used a cross-check dietary history method adapted to the Dutch setting (23). The validity and reproducibility of this method is well established (23, 24). The procedure included a 1-h interview with the participant and the person who prepared the food, the result of which was verified with the quantities of food purchased for the family during a week. On the basis of these data, the daily folate and alcohol intake were estimated using a computerized version of the Netherlands food table. For the estimation of folate intake, recent liquid chromatography data for the folate content of foods were used (25).

Longitudinal Disease Ascertainment. Information on vital status of the participants until January 1995 was obtained from municipal population registries. One man was lost to follow-up in 1989, and 3 men were lost to follow-up in 1991 because they had moved abroad or moved with unknown destination. These men were included in the analyses but censored at July 20, 1989, or December 31, 1990. The incidence of nonfatal cancer during follow-up was recorded at examinations in 1990 and 1995 using standardized questionnaires for responders and an additional short disease questionnaire for nonresponders. Data on incidence of all cancers were verified with hospital discharge data and written information from the general practitioner. Information on fatal cases of cancer was obtained from the Netherlands Central Bureau of Statistics for deaths that occurred between the baseline assessment and June 1990 and from the subjects' general practitioners for deaths that occurred thereafter. Cancer deaths were verified using hospital data. A single physician coded death from cancer according to the ninth revision of the International Classification of Diseases. Death from any cancer was defined by the ninth revision of the International Classification of Diseases codes 140–209 from lung cancer by 162, from prostate cancer by 185, from colorectal cancer by 153–154, and from kidney/bladder cancer by 188–189. The development of cancer at different sites in patients was assumed to be independent unless recorded as metastasis.

Biochemical Measurements, DNA Extraction, and Genotyping. Nonfasting venous blood samples were taken and serum stored at −20°C. No cells were stored. Serum total homocysteine was measured as described previously (26). Genomic DNA was extracted from 400 μl of serum using the QIAamp DNA blood mini kit (Qiagen) and dissolved in 200 μl of 10 mM Tris/0.1 mM EDTA. The DNA yield from serum was too low to allow direct PCR amplification. Therefore, 10 μl of the DNA solution was used in a whole genome PCR amplification using a mixture of 15-base random oligonucleotides (27). Next, *MTHFR* genotypes were determined on 2 μl of the 50-μl whole genome amplification product by PCR amplification of a 198-bp fragment containing the Ala^{222}/Val polymorphism followed by digestion with *Hin*fI as described previously (9). Two observers independently assessed all *MTHFR* genotypes, and samples were reamplified if differences were observed. The reliability of the genotypes obtained from DNA extracted from serum was checked in three ways. First, 1 of every 36 DNA extractions was a negative control using water instead of serum. None of these gave a positive signal after PCR amplification. Second, 20% of the samples were also genotyped by performing a double PCR amplification using the standard genotyping protocol thus circumventing the whole genome amplification step. No differences were observed. Third, blood samples collected in 1990 were available for 14% of the population. DNA was extracted from lymphocytes, and *MTHFR* genotypes were determined using the standard genotyping protocol. These genotypes were compared with the genotypes obtained from DNA extracted from serum that was subjected to a whole genome amplification step. Four inconsistencies were observed (correspondence rate 97%), which may be because of PCR artifacts. It, however, cannot be excluded that these were caused by logistical problems because blood for genomic DNA extraction was collected 5 years later than the serum samples (1990 and 1985, respectively). It is important to note that the differences did not follow a specific pattern. Such random misclassification is expected to lead to underestimating the strength of associations and not to false-positive associations.

Statistical Analysis. Differences in baseline characteristics according to *MTHFR* genotype were evaluated using ANOVA for normally distributed variables, the Kruskal-Wallis test for variables with a skewed distribution and an overall χ^2 test for categorical variables. Odds ratios for cancer at baseline were estimated using logistic regression. In the follow-up study, associations between *MTHFR* genotypes and cancer risk were tested using Cox proportional hazards models. For evaluating the previously suggested interaction between the *MTHFR* genotype and the intake of folate and alcohol (18, 19), prevalent and incident cases of cancer were combined to increase the power of the analysis. Prevalent and incident cases had a similar folate ($P = 0.26$) and alcohol ($P = 0.62$) intake, which indicates that no major changes in diet occurred after the diagnosis of cancer. Moreover, possible changes in diet toward higher folate and lower alcohol intakes would lead to an underestimation of the interaction. Subjects were divided according to the tertile of folate and alcohol intake, and subjects in the two highest tertiles of folate intake and the two lowest tertiles of alcohol intake were grouped. Grouping of tertiles was done on the basis of previous studies indicating that the Val/Val genotype was associated with elevated plasma homocysteine at very low intake of folate only (28) and that the Val/Val genotype was associated with an increased risk of colorectal cancer in the high alcohol consumption group and with a decreased risk in the two lower alcohol consumption groups (18). To evaluate the influence of age, the same approach was used but now the subjects were divided according to the median age. All tests were two-sided and values of $P < 0.05$ were considered statistically significant. The analyses were performed using SAS version 6.12.

RESULTS

Table 1 shows the baseline characteristics of the studied cohort of 860 men ages 65–84 years according to *MTHFR* genotype. The *MTHFR* genotype distribution was 49.4% (Ala/Ala), 42.1% (Ala/Val), and 8.5% (Val/Val) and was in Hardy-Weinberg equilibrium. The distributions of risk factors for cancer were similar for the different *MTHFR* genotypes.

As expected, the Val/Val genotype was strongly associated with

Table 1 *Baseline characteristics of men ages 65–84 years according to MTHFR genotype*[a]

	MTHFR genotype			
	Ala/Ala	Ala/Val	Val/Val	*P*
Number	425	362	73	
Age (yr)[b]	71.5 (5.3)	71.4 (5.5)	71.2 (5.3)	0.90
Body mass index, kg/m^2	25.5 (3.2)	25.5 (3.2)	25.2 (2.9)	0.75
Smoking status[c]				0.36
Current smokers, %	29.5	30.9	32.9	
Former smokers, %	50.9	50.3	57.5	
Never smokers, %	19.6	18.8	9.6	
Alcohol intake, g/day[d]	13.5 (17.4)	12.7 (16.4)	16.5 (18.4)	0.24
Folate intake, μg/day[d]	201 (61)	202 (63)	192 (55)	0.49

[a] Values in parentheses for variables are SDs.
[b] Range 65–84 years.
[c] Missing data on smoking for 1 of 860 subjects.
[d] Missing data on folate and alcohol intake for 56 of 860 subjects.

elevated total plasma homocysteine levels ($P = 0.0001$; Table 2). For subjects homozygous for the Ala-allele, the mean level was 14.8 nmol/ml, whereas it was 21.6 nmol/ml among subjects homozygous for the Val-allele (Table 2). This association was significantly modulated by folate intake (P, interaction = 0.0006; Table 2).

The baseline prevalence of cancer among subjects homozygous for the Val-allele (11.0%) tended to be higher than among those homozygous for the Ala-allele (6.1%), although this was not statistical significant ($P = 0.17$; data not shown). The risk of cancer associated with the Val/Val genotype as estimated with the odds ratio was 1.95 (95% CI, 0.84–4.54) compared with the Ala/Ala genotype.

During the 10-year follow-up period, 149 new cases of cancer occurred among the 793 men without cancer at baseline. The most common sites where cancer developed were the lung (27%), prostate (13%), colorectum (11%), and the kidney or bladder (10%). Compared with men homozygous for the common Ala-allele, the age-adjusted risk of cancer was 1.80-fold (95% CI, 1.09–3.00) increased among men with the Val/Val genotype (Table 3). Additional adjustment for smoking status (RR, 1.77; 95% CI, 1.06–2.94) or smoking status, body mass index, and alcohol and folate intake (RR, 1.64; 95% CI, 0.96–2.80; missing data for 54 subjects) did not appreciably alter this risk estimate. The analysis of the four common forms of cancer revealed that the higher incidence of cancer was because of significantly increased risks of cancer of the prostate, colorectum, and kidney or bladder, whereas the risk of lung cancer remained unaffected (Table 3).

On the basis of previously published reports (18, 19), we next evaluated the influence of folate and alcohol intake on the association between the Val/Val genotype and cancer risk (Table 4). To increase the power of the analysis we combined prevalent ($n = 67$) and incident ($n = 149$) cases of cancer (see "Materials and Methods").

Table 2 *The relationship of MTHFR genotypes with total plasma homocysteine levels depending on folate intake*[a]

Folate intake	*N*	Homocysteine[a] according to *MTHFR* genotype			*P*
		Ala/Ala	Ala/Val	Val/Val	
All subjects	804	14.8 (0.4)	15.5 (0.4)	21.6 (0.9)	0.0001
Folate intake tertiles					
<169.8 μg/day	268	15.6 (0.7)	17.2 (0.7)	25.1 (1.4)	0.0001
169.8–214.8 μg/day	267	15.0 (0.7)	14.6 (0.7)	24.3 (1.8)	0.0001
>214.8 μg/day	269	13.6 (0.7)	14.7 (0.7)	14.7 (1.6)	0.38
				Interaction	0.0006

[a] Values in parentheses for variables are SEs. Folate intake is estimated as described in "Materials and Methods."
[b] In μmol/liter.

Table 3 *RRs of cancer according to MTHFR genotype among subjects without cancer at baseline between 1985 and 1995*[a]

End point	*MTHFR* genotype		
	Ala/Ala	Ala/Val	Val/Val
All cancers			
Cases/at risk	71/399	59/329	19/65
RR	1 (reference)	0.99 (0.70–1.40)	1.80 (1.09–3.00)
Lung cancer			
Cases/at risk	23/399	17/329	4/65
RR	1 (reference)	0.86 (0.46–1.61)	1.15 (0.40–3.33)
Prostate cancer			
Cases/at risk	8/399	9/329	4/65
RR	1 (reference)	1.24 (0.48–3.22)	3.48 (1.05–11.6)
Colorectal cancer			
Cases/at risk	7/399	7/329	4/65
RR	1 (reference)	1.16 (0.41–3.30)	3.65 (1.07–12.5)
Kidney and bladder cancer			
Cases/at risk	6/399	5/329	5/65
RR	1 (reference)	0.98 (0.30–3.20)	5.48 (1.67–18.0)

[a] RRs are adjusted for age. Values in parentheses are 95% CIs.

Table 4 *Odds ratios for cancer depending on folate intake, alcohol intake, and age according to MTHFR genotype*

Subgroup	*MTHFR* genotype		
	Ala/Ala	Ala/Val	Val/Val
Folate intake[b,c]			
≤169.8 μg/day			
Cases/at risk	30/131	30/110	11/27
RR	1 (reference)	1.30 (0.72–2.37)	2.64 (1.08–6.41)
>169.8 μg/day			
Cases/at risk	63/261	59/236	13/39
RR	1 (reference)	1.04 (0.69–1.57)	1.60 (0.77–3.30)
Alcohol intake[c,d]			
≤14 g/day			
Cases/at risk	62/267	55/242	12/37
RR	1 (reference)	0.99 (0.65–1.50)	1.63 (0.77–3.43)
>14 g/day			
Cases/at risk	31/125	34/104	12/29
RR	1 (reference)	1.35 (0.75–2.44)	2.26 (0.95–5.40)
Age[e]			
≤71 years			
Cases/at risk	51/228	40/203	14/47
RR	1 (reference)	0.87 (0.55–1.40)	1.37 (0.68–2.97)
>71 years			
Cases/at risk	46/197	52/159	13/26
RR	1 (reference)	1.57 (0.98–2.51)	3.14 (1.35–7.28)

[a] In these analyses, prevalent and incident cases of cancer are combined (see "Materials and Methods"). Odds ratios are adjusted for age. Values in parentheses are 95% CIs.
[b] Stratified according to lowest tertile of folate intake and the two highest tertiles.
[c] For 56 subjects data on alcohol and folate intake were missing.
[d] Stratified according to two lowest tertiles of alcohol intake and highest tertile.
[e] Subjects are stratified according to median age.

Among men whose folate intake was ≤169.8 μg/day, the Val/Val genotype was associated with a 2.64-fold increased risk of cancer as compared with a 1.60-fold increased risk among men with a higher folate intake. Among men who consumed >14 g of alcohol/day, the Val/Val genotype was associated with a 2.27-fold increased risk of cancer as compared with a 1.63-fold increased risk among men with a lower alcohol consumption. Furthermore, the risk of cancer associated with the Val/Val genotype was 3.14-fold increased among men older than the median age of 71 years, whereas the risk was only 1.37-fold increased among younger men (Table 4).

DISCUSSION

We studied the influence of a common *MTHFR* Ala/Val polymorphism on the risk of cancer in a population-based cohort of men ages 65–84 years old. Consistent with the hypothesis that the Val/Val genotype contributes to a disturbed folate metabolism *in vivo*, the genotype was associated with considerably elevated levels of plasma homocysteine, particularly among those with lower folate intakes. Over a 10-year follow-up period, the Val/Val genotype was associated with a ~2-fold increased risk of cancer. A similar 2-fold increase in risk was observed in the comparison of cancer at baseline, although this was not statistically significant.

Investigation of common forms of cancer revealed that, although the numbers were small, the incidences of cancer of the prostate, the colorectum and the kidney or bladder were significantly increased among men with the Val/Val genotype, whereas the risk of lung cancer was not affected possibly because the large effect of smoking overrides milder risk factors. The latter finding is in agreement with our unpublished results that the Val/Val genotype frequency was not increased among 211 lung cancer patients (11.4%) as compared with 250 controls (12.4%) and a previous report (29).

Our finding that the *MTHFR* variant is associated with an increased risk of cancer in elderly men from the general population confirms our earlier observation in a population-based study among subjects

ages ≥ 85 years (14). It is also in line with the subsequently reported association of the Val/Val genotype with an increased prevalence of gastric cancer (15), colorectal cancer with microsatellite instability (16), and cervical dysplasia (17). In contrast, several studies observed a protective effect of the Val/Val genotype on the risk of colorectal cancer (18, 19) and acute lymphocytic leukemia (20, 21). The protective effect of the Val/Val genotype was attributed to an associated diversion of folate to the synthesis of dTMP from dUTP. However, the Val/Val genotype did not confer higher resistance to uracil misincorparation into DNA *in vitro* (13), challenging this explanation. The opposite, deleterious effect of the Val/Val genotype as observed in our current and previous studies may arise as a consequence of the depletion of the product of MTHFR, 5-methylTHF (10), which is vital for DNA methylation reactions. The Val/Val genotype was indeed shown to be associated with decreased genomic DNA methylation in particular among subjects with a low folate status (12).

Whether the protective or the deleterious effect of the Val/Val genotype prevails may depend on environmental factors. The reduced risk of colorectal cancer associated with the Val/Val genotype found in previous studies was abolished or even reversed among men with lower folate status or higher alcohol intake (18, 19). Alcohol interferes with folate absorption and usage (30, 31), and alcohol is a methyl group antagonist (32). Similarly, our analyses indicated that the cancer risk was particularly increased among men with a lower folate or higher alcohol intake. The protective associations with colorectal cancer were observed in male American health professionals and physicians who are considered to be health conscious and well nourished and are commonly using vitamin supplements (18, 19). Consequently, the median folate intake of the health professionals was ~2-fold higher than that of the participants in our population-based study (18). It may be hypothesized that such factors suppressed the deleterious effects of the Val/Val genotype. Additionally, age differences may play a role. Our analyses suggested that the adverse effects of the Val/Val genotype increase with age. The subjects in our current and previous (14) study were 65–84 and 85–100 years old, respectively, which is substantially higher than those in the studies on colorectal cancer [40–75 (18 subjects) and 40–84 (19 subjects) years] and leukemia [16–70 (20 subjects) and younger than 15 (21 subjects) years].

The *MTHFR* gene is located on chromosome 1p36.3. This locus was implicated in prostate cancer by several genome-wide linkage scans (33–35). It would be interesting to test whether variation in the *MTHFR* gene had contributed to these outcomes.

In conclusion, our current and previous studies in two independent populations indicate that homozygosity for the *MTHFR* Ala/Val polymorphism increases the risk of cancer in elderly men from the general population. High folate and low alcohol intake and a younger age may help to suppress the deleterious effect on cancer risk. The mechanism underlying this association may involve a decreased DNA methylation as a result of a disturbed folate metabolism. We hypothesize that genome-wide hypomethylation promoting genomic instability (5) rather than gene-specific effects such as derepression of proto-oncogenes (3, 4) is relevant in this respect.

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