A Common Mutation in the Methylenetetrahydrofolate Reductase Gene Is a Determinant of Hyperhomocysteinemia in Epileptic Patients Receiving Anticonvulsants

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Hyperhomocysteinemia is a condition caused by both genetic and nongenetic factors. To determine whether a common methylenetetrahydrofolate reductase (MTHFR) variant is related to elevated homocysteine concentrations in epileptic patients receiving anticonvulsants, we investigated the plasma total homocysteine (tHcy) level, folate level, and MTHFR 677 C → T mutation using a polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis with *HinfI* digestion in 103 patients with epilepsy and 103 normal controls. The prevalence of hyperhomocysteinemia (≥ 11.4 μmol/L, 90th percentile of control group) was higher in patients than in controls (25% *v* 10.0%, *P* = .007). The homozygosity for the 677 C → T mutation of MTHFR was associated with elevated tHcy and low folate levels. The magnitude of hyperhomocysteinemia in MTHFR TT homozygotes was more pronounced in epileptic patients than in controls (18.2 ± 1.6 *v* 9.1 ± 1.2 μmol/L, *P* = .04). In epileptic patients, hyperhomocysteinemia was more frequent in MTHFR TT genotypes versus CT or CC genotypes (58% *v* 17% and 16%, *P* < .001). Multiple logistic regression analysis showed that MTHFR TT genotype was an independent predictor of hyperhomocysteinemia in epileptic patients receiving anticonvulsants (phenytoin and carbamazepine but not valproic acid), suggesting that gene-drug interactions induce hyperhomocysteinemia. These findings indicate that epileptic patients receiving anticonvulsants may have a higher folate requirement to maintain a normal tHcy level, especially homozygotes for MTHFR 677 C → T mutation.

IT IS KNOWN that patients taking anticonvulsants such as phenytoin, phenobarbital, primidone,[1] and carbamazepine,[2,3] are susceptible to folate deficiencies. The effects of valproic acid on folate metabolism have also been implicated.[4] However, the mechanisms by which anticonvulsants induce folate deficiency are not clear. In the mouse model, Billings[5] found that long-term phenytoin treatment decreased the plasma folate level and inhibited the enzyme activity of 5,10-methylenetetrahydrofolate reductase (MTHFR), which catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. In humans, because 5-methyltetrahydrofolate, a major circulating form of folate, is the primary methyl donor for remethylation of homocysteine to methionine,[6] a low folate status is associated with elevated total homocysteine (tHcy) concentrations. These considerations raise the possibility that the combination of folate and MTHFR enzyme deficiency and anticonvulsant treatment may decrease the remethylation of homocysteine. thus inducing hyperhomocysteinemia. Epidemiological studies have shown that moderate elevations of plasma tHcy are independently associated with coronary artery disease, stroke, peripheral arterial occlusive disease, venous thromboembolism, and vascular dementia.[7-11] In particular, homocysteine may have excitotoxicity to neurons as agonists of *N*-methyl-D-aspartate (NMDA) receptors.[12]

Hyperhomocysteinemia in the absence of renal dysfunction indicates disrupted sulfur amino acid metabolism, and can be derived from genetic defects, environmental factors such as vitamin deficiency and drugs, or a combination of both.[8,9] Folate status is a major environmental determinant of hyperhomocysteinemia in patients with vascular disease, as well as healthy controls.[6,8]

A mutation of MTHFR with thermolability was found in 17% of North American patients with coronary artery disease and in 5% of normal subjects.[13] Frosst et al[14] found a missense 677 C → T mutation, in which a cytidine residue at nucleotide position 677 in the gene coding for the MTHFR enzyme is replaced by thymidine. The homozygous MTHFR 677 C → T mutation conferred a specific activity of 50% of the normal mean activity with enhanced thermolability.[14] Indeed, this mutation appears to be responsible for most cases of thermolabile MTHFR, except for a small number of compound heterozygotes (S.S. Kang, personal communication, August 1997).

Recently, Ono et al[15] reported that epileptic patients taking anticonvulsants had a low folate status and increased levels of tHcy in a population composed mostly of Japanese children. However, there were no reports on either the prevalence of hyperhomocysteinemia in adult patients or the effects of genetic enzyme defects on tHcy concentration in epileptic patients receiving anticonvulsants. Since homozygosity of the thermolabile MTHFR mutant allele occurs commonly in 5% to 15% of the various populations,[9] we postulated that the existence of homozygotes for the mutant allele of MTHFR predisposes epileptic patients to elevated tHcy and further augments the severity of hyperhomocysteinemia in epileptic patients receiving anticonvulsants.

In the present study, we report that elevated tHcy levels are a common finding in epileptic patients receiving anticonvulsant treatments, and that a MTHFR 677 C → T homozygous variant is a major determinant of hyperhomocysteinemia in epileptic patients receiving anticonvulsant treatments.

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SUBJECTS AND METHODS

Subjects

Subjects were consecutively recruited from epileptic patients who were under treatment with one or two anticonvulsants at Samsung Medical Center, Seoul, Korea, from March through July 1997. The anticonvulsants used in the study group were as follows: phenytoin (n = 18), carbamazepine (n = 23), valproic acid (n = 33), phenytoin and carbamazepine (n = 5), phenytoin and valproic acid (n = 11), and carbamazepine and valproic acid (n = 9). One hundred six patients with epilepsy between the ages of 15 and 40 years consented to participate in the study. Three patients with thyroid or renal dysfunction (serum creatinine ≥ 1.5 mg/dL) were excluded. No patients showed evidence of anemia, neutropenia, thrombocytopenia, or decreased serum albumin. All patients were on a regular diet and had no evidence of debilitating diseases. Serum levels of antiepileptics were in the therapeutic range. One hundred three patients (60 men and 43 women) were eligible for inclusion, four of whom were taking folate tablets (1,000 μg/d) with anticonvulsant treatment.

The control group was recruited from healthy subjects who visited the Family Practice Center for health examinations during the study period. Subjects between the ages of 15 and 40 years who had no evidence of occlusive vascular disease and no birth defects in their offspring were consecutively selected. The subjects were matched according to gender and age within 5 years. Exclusion criteria were identical to those of the patient group. Multivitamin users were also excluded from the study. A total of 103 subjects (60 men and 43 women) were recruited for the control group. Informed consent was obtained from all subjects. The study was approved by the Institutional Review Board.

Determination of Plasma Homocysteine and Folate Concentrations

Blood specimens were collected from the antecubital vein in Vacutainer K3 EDTA tubes (Becton Dickinson, Franklin Lakes, NJ) from patients and control subjects in the nonfasting state. The tubes were immersed in ice water and transported to the laboratory at a temperature of 0°C. Blood samples were centrifuged (3,000 × *g* for 5 minutes) at 4°C within 3 hours and stored with aliquots of plasma and blood cells at −75°C until analysis. Samples from the patients and controls were stored for the same duration and were analyzed at the same time.

The tHcy concentration was determined using the high-performance liquid chromatography (HPLC) method of Vester and Rasmussen.[16] Tri-*n*-butylphosphine, derivatizing agent, mercaptopropionylglycine, and DL-homocysteine were obtained from Sigma Chemical (St Louis, MO). For the HPLC system, a Waters (Milford, MA) autosampler and pumps and Waters 474 scanning fluorescence detector system equipped with a Merck LiChrospher 100 RP-18 column (4 × 125 mm, 5-μm particles) and Merck LiChrospher 100 RP-18 guard column (Merck, Darmstadt, Germany) were used. Plasma folate and vitamin B_{12} were determined by a radioimmunoassay (Diagnostic Products Corp, Los Angeles, CA). The intraassay and interassay coefficients of variation for tHcy concentrations were 3.5% and 5.4%.

Identification of MTHFR Genotype

Genomic DNA was extracted from peripheral blood cells by the standard method. DNA samples were amplified by a polymerase chain reaction (PCR) using 2.5 U Taq polymerase (Takara, Shiga Otsu, Japan), 2 mmol/L magnesium chloride, 200 μmol/L dNTPs, and 150 ng oligonucleotide primer in each. Amplification was performed using 55°C as the annealing temperature for 2 minutes, 72°C extension temperature for 3 minutes, and 95°C denaturation temperature for 1 minute in a model 9600 thermocycler (Perkin Elmer, Norwalk, CT). PCR primer sequences[14] were 5′-CAA-AGG-CCA-CCC-CGA-AGC-3′ for sense primers and 5′-AGG-ACG-GTG-CGG-TGA-GAG-TG-3′ for antisense primers. After 32 cycles, specific bands for PCR products were identified by electrophoresis and the PCR products were then digested by the enzyme *Hin*fI (Takara) at 37°C, because the nucleotide 677 mutation creates a restriction site for *Hin*fI. The restriction fragments were separated by electrophoresis in 3:1 NuSieve Gel from FMC BioProducts (Rockland, ME) and ethidium bromide staining. PCR results were identified regardless of patient-control status. Mutant alleles were designated as "T" and the wild-type allele as "C."

Statistics

Continuous variables are expressed as the mean ± SD. The χ^2 test was used for categorical variables, and Student's *t* test or Mann-Whitney *U* test were used for continuous variables. Since tHcy levels were not in normal distribution, logarithmic transformation was used. The mean values for MTHFR genotypes were tested by ANOVA. A logistic regression model was used to calculate the odds ratio for the MTHFR variant in hyperhomocysteinemia. Associations in continuous variables were tested by calculating Pearson's correlation coefficients. The cutoff point of hyperhomocysteinemia was 11.4 μmol/L, which represents the 90th percentile for the distribution of tHcy in the control group.

RESULTS

The mean levels of tHcy, folate, and vitamin B_{12} in 99 epileptic patients and 103 healthy subjects aged 15 to 40 years are shown in Table 1. The mean tHcy was higher in the patients versus the controls. There were no subjects with folate deficiency (≤2.0 ng/mL) and no difference in the mean folate level between case and control groups. Vitamin B_{12} levels were in the normal range and were higher in the patients versus controls ($P < .001$). The prevalence of hyperhomocysteinemia, defined by the 90th percentile for the control group (11.4 μmol/L), was 2.5-fold higher in epileptic patients versus controls (Table 1).

Distribution of MTHFR Genotypes in Epileptic and Healthy Subjects

The homozygosity for MTHFR 677 C → T mutation was more frequent in epileptic patients (24 of 103) compared with healthy controls (13 of 103, $P < .05$; Table 2). According to medication, the frequency of MTHFR TT genotype was four (22.2%) in epileptic patients with phenytoin, eight (34.8%) with

Table 1. Clinical and Biochemical Characteristics of Epileptic Patients Receiving Anticonvulsants and Healthy Controls

Variable	Epileptic Patients (n = 99)	Healthy Controls (n = 103)
Sex ratio (male/female)	60/39	60/43
Age (yr)	27.5 ± 8.5	28.1 ± 9.8
Duration (yr)	6.4 ± 4.1	0
tHcy (μmol/L)	11.2 ± 1.5*	7.9 ± 1.2
Folate (ng/mL)	8.3 ± 4.5	9.0 ± 4.1
Vitamin B_{12} (pg/mL)	854 ± 342*	580 ± 204
HHcy (%)	25.0*	10.0

NOTE. HHcy, tHcy ≥11.4 μmol/L, defined as the 90th percentile for the control group. Four women on folic acid supplementation are shown in Table 3. tHcy values were logarithmically transformed. A χ^2 test for categorical variables and Student's *t* test for continuous variables were used.

*$P < .05$.

Table 2. Comparison of tHcy and Plasma Folate Between Epileptic Patients Receiving Anticonvulsants and Healthy Controls According to MTHFR Genotype

Genotype	Epileptic Patients (n = 103)	Healthy Controls (n = 103)
TT	(n = 24)	(n = 13)
tHcy (μmol/L)	18 2 ± 1.6*	9 1 ± 1.2
Folate (ng/mL)	7.4 ± 4.9	8.1 ± 2.3
CT	(n = 54)	(n = 53)
tHcy (μmol/L)	8.6 ± 1.3	7.6 ± 1.2
Folate (ng/mL)	8.6 ± 3.9	9.3 ± 4.0
CC	(n = 25)	(n = 37)
tHcy (μmol/L)	9.2 ± 1.4	7.9 ± 1.1
Folate (ng/mL)	8.5 ± 5.1	9.0 ± 4.6

NOTE. tHcy was logarithmically transformed. Values from 4 women on folic acid supplementation are not included.
*$P < .05$.

carbamazepine (n = 23), and five (15.2%) with valproic acid. Four patients who were taking folate (1,000 μg/d) with anticonvulsant treatment had MTHFR TT genotypes.

Correlation Between tHcy, Plasma Folate, and Vitamin B_{12} Concentrations

Homocysteine levels exhibited an inverse correlation with plasma folate in patients with epilepsy ($r = -.28$, $P = .007$) and in controls ($r = -.28$, $P = .0001$), but had no correlation with vitamin B_{12}. There was no correlation between folate, vitamin B_{12}, and the age of patients and controls.

Relationship Among tHcy, Folate, and MTHFR Genotypes

In epileptic patients with MTHFR TT genotype, the mean tHcy level was significantly higher than the level in CT or CC genotypes (18.2 ± 1.6 *v* 8.6 ± 1.3 and 9.2 ± 1.4 μmol/L, $P = .0003$). The mean plasma folate level was not significantly lower in TT genotypes compared with CT and CC genotypes (7.4 ± 4.9 *v* 8.6 ± 3.9 and 8.5 ± 5.1 ng/dL, $P > .05$). The mean tHcy level in MTHFR TT homozygotes was higher in epileptic patients versus controls (18.2 ± 1.6 *v* 9.1 ± 1.2 μmol/L, $P = .04$) (Table 2).

In epileptic patients receiving anticonvulsants, hyperhomocysteinemia was more frequent in the MTHFR TT genotype versus CT or CC genotypes (58% *v* 17% and 16%, $P < .001$).

Effect of Folate Supplementation on tHcy Concentrations in Epileptic Patients Homozygous for 677 C → T Mutation in MTHFR Gene

The magnitude of hyperhomocysteinemia for patients with MTHFR TT genotypes was twofold higher than for the controls (Table 2). However, in MTHFR TT homozygotes, epileptic patients receiving folate supplementation had a remarkably lower tHcy level than those who did not receive folate supplementation (4.9 ± 0.1 *v* 19.7 ± 1.6 μmol/L, $P = .01$) and a higher folate level (Table 3).

Effect of MTHFR Genotype on tHcy and Plasma Folate According to Anticonvulsant Treatment

In patients receiving phenytoin or carbamazepine, the MTHFR TT genotype group showed higher homocysteine levels (Fig 1) and lower folate levels (6.3 ± 2.2 and 6.5 ± 2.1 *v* 8.7 ± 0.4 and 8.1 ± 2.3 ng/mL, $P < .05$) compared with patients receiving valproic acid or control subjects. However, as a whole, there was no difference in folate levels between patients and controls (Table 4).

When multiple logistic regression analysis was performed, MTHFR TT genotype emerged as a significant predictor of hyperhomocysteinemia in epileptic patients, with an odds ratio of 7.3 (95% confidence interval, 3.7 to 13.5, $P < .001$), adjusted for the effects of medication (phenytoin and carbamazepine *v* valproic acid), gender, and duration of medication (5 years). However, medication, gender, and duration of medication did not remain significant ($P > .05$).

DISCUSSION

An elevated plasma level of homocysteine can be induced by either genetic or environmental factors. Because MTHFR 677 C → T mutation is known to be associated with reduced enzyme activity and anticonvulsants decrease MTHFR enzyme activity, we examined whether the combined presence of MTHFR 677 C → T mutation and anticonvulsants in epileptic patients causes elevated tHcy. We observed a higher mean tHcy and higher prevalence of hyperhomocysteinemia in epileptic patients receiving anticonvulsants than in the controls. Moreover, in the population studied, the frequency of homozygosity for MTHFR 677 C → T mutation in epileptic patients was higher than that in the controls. We also found that epileptic patients with MTHFR TT homozygotes had a higher tHcy and lower plasma folate level compared with heterozygotes or wild-types in the phenytoin and carbamazepine group, but not in the valproic acid group.

Several mechanisms have been proposed to account for the effect of long-term anticonvulsant therapy on folate deficiency: reduced intestinal absorption, increased metabolism in liver, increased metabolic requirement for folate as a cofactor for hydroxylation of the anticonvulsant, and altered activity of some enzymes involved in one-carbon transfer.[1] However, these are still uncertain. Phenytoin and carbamazepine each induce hepatic microsomal enzymes, whereas valproic acid and zonisamide do not. Kishi et al[3] demonstrated that the enzyme-inducing anticonvulsants reduced serum folate levels while the non–enzyme-inducing agents did not, thus suggesting that the induction of microsomal liver enzymes may be associated with a reduction of serum folate. The effect of valproic acid on folate metabolism has been demonstrated in the rat[4] and in humans,[2,3] but it has not been fully elucidated. In the mouse model, phenytoin treatment decreased plasma folate and inhibited the enzyme activity of MTHFR.[5] Our findings are consistent with

Table 3. Comparison of tHcy and Folate Levels Between Epileptic Patients Homozygous for MTHFR 677 C → T Mutation With and Without Folate Supplementation

Variable	Epileptic Patients Receiving Anticonvulsants	
	With Folate (n = 4)	Without Folate (n = 20)
tHcy (μmol/L)	4.9 ± 0.1*	19.7 ± 1.6
Folate (ng/mL)	17.6 ± 12.4*	6.5 ± 2.9
Vitamin B_{12} (pg/mL)	611.0 ± 455 4	803.1 ± 315.6

*$P < .05$, Mann-Whitney *U* test

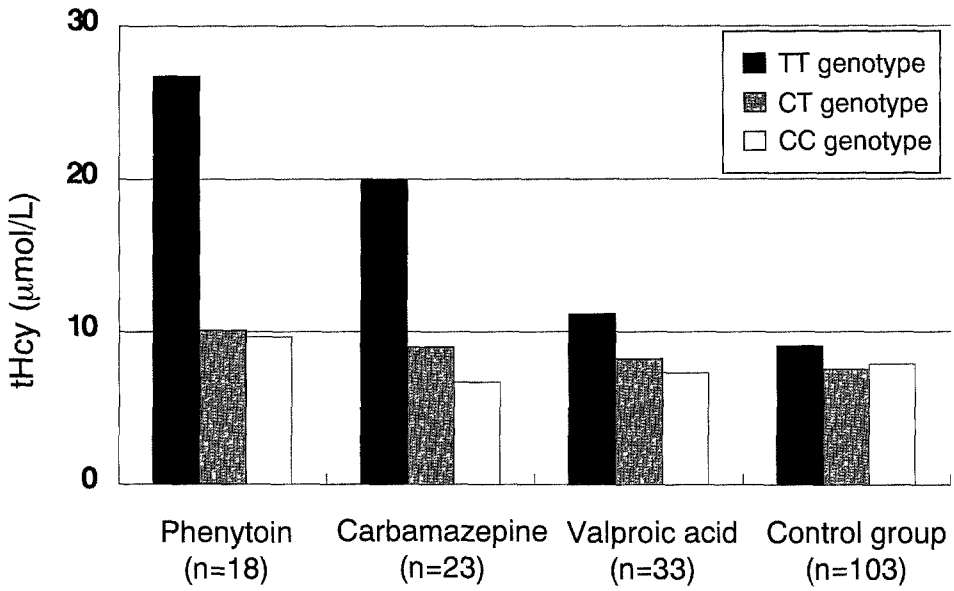

Fig 1. Mean tHcy levels in epileptic patients receiving phenytoin, carbamazepine, or valproic acid and the control group according to MTHFR genotype.

the observation in mice, and suggest that the effect of valproic acid on folate metabolism may not be related to the MTHFR reaction.

Prior studies reported a low folate status in epileptic patients receiving anticonvulsants.[2,3,17,18] However, in our study, only patients with the MTHFR TT genotype receiving phenytoin and carbamazepine showed significantly lower folate levels compared with the controls and valproic acid group. Ono et al[15] reported that elevated levels of homocysteine in pediatric patients on anticonvulsants are associated with the duration of anticonvulsant treatment. In our study, there was no difference in mean tHcy between patients on anticonvulsants for more than 5 years versus less than 5 years (10.6 ± 4.9 *v* 9.2 ± 4.7 μmol/L, $P > .05$).

The high frequency of homozygosity for MTHFR 677 C → T mutation in patients with epilepsy may have an implication for various diseases. It is not clear whether the impaired homocysteine metabolism is related to epileptogenesis or to alteration of the seizure threshold. Lipton et al[12] showed that homocysteine acts as an agonist at the glutamate binding site of the NMDA receptor. In the rat model, it was demonstrated that NMDA receptor antagonist is protective against seizures induced by homocysteine.[19] The possible role of MTHFR mutation and homocysteine in epileptogenesis should be studied further.

Table 4. Comparison of tHcy Level, Proportion of Hyperhomocysteinemia, and Plasma Folate Level Between Patients Receiving Anticonvulsants and Healthy Controls

Variable	Epileptic Patients			Healthy Controls (n = 103)
	Phenytoin (n = 18)	Carbamazepine (n = 23)	Valproic Acid (n = 33)	
Sex ratio (male/female)	12/6	11/12	19/14	60/43
Age (yr)	28.5 ± 7.6	27.5 ± 9.9	26.8 ± 10.1	28.1 ± 9.8
Duration (yr)	9.8 ± 4.5	3.8 ± 3.5	5.5 ± 2.9	—
tHcy (μmol/L)	14.6 ± 1.6*	12.7 ± 1.7*	7.9 ± 1.2	7.9 ± 1.2
Folate (ng/mL)	7.9 ± 4.0	7.0 ± 2.8	10.7 ± 4.5	9.0 ± 4.1
HHcy (%)	50.0*	30.4*	12.1	10.0

NOTE. A χ^2 test for categorical variables and Student's *t* test for continuous variables were used for comparisons between patients and control group.

*$P < .05$.

The clinical consequences of low folate status in epileptic patients receiving anticonvulsant therapy remain to be established. Fröscher et al[17] observed that patients with low folate concentrations had more frequent mental problems than those with normal folate levels. Their cognitive and affective states were improved with folate supplementation. Folate deficiency can lead to megaloblastic anemia, but it rarely occurs in clinical settings. With respect to folate status, extensive studies have focused on the development of hyperhomocysteinemia, recognized as an independent risk factor for occlusive vascular disease.[7-10]

Patients with epilepsy are subject to an increased risk of premature death from underlying causes or from epilepsy itself. Stroke is one of the most common causes of death in epileptic patients.[20] Hyperhomocysteinemia is associated with increased risk of stroke[10] and coronary artery disease.[7] MTHFR TT genotype is associated with hyperhomocysteinemia of patients with coronary artery disease or stroke.[11,21,22] Since the dominant cause of seizures in the elderly is a previous stroke accompanied by concurrent coronary artery disease, anticonvulsant treatment may increase the risk of recurrent strokes or heart attacks through elevated of tHcy. Even in normal subjects with apparently adequate nutritional folate status, older patients had elevated homocysteine levels.[23]

van der Put et al[24] showed that MTHFR 677 C → T mutation is a genetic risk factor for spina bifida. Elevated tHcy levels in pregnant women also increased the risk for neural tube defects in their offspring.[25] The incidence of malformations in infants of mothers with epilepsy treated with anticonvulsants is two or three times greater than in infants of mothers without epilepsy.[26] The high frequency of MTHFR TT genotype and defective folate metabolism due to anticonvulsants that we observed may be associated with a high incidence of malformations in infants of mothers receiving antiepileptic treatment. Periconceptional folate supplementation is known to reduce the risk of neural

tube defects in pregnant women.[27] The presence of the common MTHFR mutation may link the occurrence of birth defects to anticonvulsant therapy and folic acid deficiency in epileptic women. This implies that the gene-drug interaction plays a role in the development of the diseases, including neural tube defects, in the offspring of epileptic women. In a clinical trial, folic acid supplementation decreased plasma homocysteine levels remarkably in subjects with hyperhomocysteinemia and also in MTHFR TT homozygotes.[28] We also observed that epileptic patients receiving folic acid supplements had low homocysteine and high folate status.

In conclusion, moderate hyperhomocysteinemia is frequently found in epileptic patients receiving anticonvulsant, and tHcy levels are inversely correlated with plasma folate levels. With respect to genetic variation, a common MTHFR 677 C → T mutation is a determinant of hyperhomocysteinemia in epileptic patients receiving anticonvulsants, suggesting that a gene-drug interaction induces hyperhomocysteinemia. Since hyperhomocysteinemia is a modifiable risk factor for vascular disease and a sensitive marker for folate status, we suggest that epileptic patients receiving anticonvulsants be monitored for tHcy levels. Epileptic patients receiving anticonvulsants may have a higher folate requirement to maintain a normal tHcy level, especially homozygotes for the MTHFR 677 C → T mutation.

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