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Genetic Determinants of Folate Status in Central Bohemia

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Summary

Although several genetic factors have been implicated as determinants of blood folate concentration in various populations, their effect on folate status in the Czech population has not yet been examined. We explored whether blood folate concentrations in healthy Czech population are associated with polymorphisms in 5,10-methylenetetrahydrofolate reductase (MTHFR), folate hydrolase 1 (FOLH1), reduced folate carrier (RFC), and folate receptor (FOLR1) genes. In a cross-sectional study of 591 control subjects we determined genotypes by PCR-RFLP or ARMS-PCR methods, and plasma and erythrocyte folates by MEIA. The effect of different genotypes on folate status was examined by non-parametric tests and by regression analysis. The prevalence of the MTHFR 677C>T, MTHFR 1298A>C, FOLH1 1561C>T, RFC 80G>A and FOLR1 480G>C variant alleles was 0.34, 0.33, 0.05, 0.44 and 0.00, respectively. Only the MTHFR 677C>T variant was significantly associated with plasma folate concentrations (median 14.7, 14.0 and 12.2 nmol/l for the CC, CT and TT genotypes, respectively). Our study showed that among the five studied allelic variants, only the 677C>T polymorphism in the MTHFR gene is a significant genetic determinant of plasma folate concentrations in Czech population.

Key words

Folates • Folate hydrolase 1 • Reduced folate carrier • 5,10-Methylenetetrahydrofolate reductase • Polymorphism

Introduction

Folates constitute a group of compounds derived from the 5,6,7,8-tetrahydropteroylglutamate, more commonly referred to as tetrahydrofolate. The biological role of folates is to facilitate the metabolism of methionine, serine, glycine, choline and histidine, the biosynthesis of purines and pyrimidines, and the assimilation or oxidation of formate (Shane 1990).

Dietary folates have to pass through a number of physiological and metabolic processes, which convert them into metabolically active forms (Fowler 2001). The predominant active circulating form of folates is the 5-methyltetrahydrofolate (Selhub and Miller 1992). Disturbances of folate metabolism are associated with many human diseases such as epithelial cancers, anemia, peripheral neuropathies, cardiovascular disease and pregnancy complications (Rimm *et al.* 1998, Eichholzer

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et al. 2001, Voutilainen *et al.* 2001, McDonald *et al.* 2003).

Folate status is influenced by many exogenous and endogenous factors. The common exogenous factors include diet, smoking, alcohol consumption and drugs, while the endogenous factors include ethnicity, functional status of bowel and liver, and the presence of genetic variants (Halsted *et al.* 2002, Ganji and Kafai 2003, Lindeman *et al.* 2003, Nakano *et al.* 2003). More than twenty genes have been implicated in folate metabolism and transport (Cook 2001). Allelic variants in only a few of these genes have been analyzed in population studies and their effects on folate status have been examined.

Two allelic variants in the 5,10-methylenetetrahydrofolate reductase gene (OMIM 607093, EC 1.5.1.20) have been examined extensively in numerous studies including the Czech population (Beneš *et al.* 2001). The MTHFR enzyme catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate (5-methylTHF). In a variety of populations homozygosity for the 677C>T polymorphism has been associated with low plasma folate levels (Van Der Put *et al.* 1995), accumulation of formylated folate forms in erytrocytes (Bagley and Selhub 1998), and with elevated concentrations of plasma total homocysteine (Frosst *et al.* 1995, Van Der Put *et al.* 1995, Rozen 2001). The 1298A>C MTHFR variant, which was analyzed in some population studies, did not substantially influence the folate concentrations (Weisberg *et al.* 1998).

Polymorphisms in the FOLH1 and RFC genes belong to the less frequently studied determinants of folate status. The enzyme folate hydrolase 1 (glutamate carboxypeptidase II, OMIM 600934, EC 3.4.17.21) hydrolyzes terminal glutamate residues in the intestine, playing thus an important role in the absorption of dietary folates (Devlin *et al.* 2000). The carriership of the 1561C>T variant was associated with lower concentrations of plasma folates in one study (Devlin *et al.* 2000) and with higher concentrations in other studies (Vargas-Martinez *et al.* 2002, Winkelmayer *et al.* 2003). The reduced folate carrier, RFC, (OMIM 600424) is an essential folate transporter (Chango *et al.* 2000). Although different genotypes in the RFC 80G>A locus were associated with varying folate concentrations, the effect was not statistically significant in several published studies (Chango *et al.* 2000, Morin *et al.* 2003, Winkelmayer et al. 2003).

The folate receptor FOLR1 (OMIM 136430), which is a folate transporter, plays an important role in the embryonic development as demonstrated by numerous malformations in mice with deleted FOLR1 gene (Finnell *et al.* 2002), and by neural tube defects in mice embryos treated by FOLR1 antisense mRNA (Hansen *et al.* 2003). Only one putative polymorphism in this gene, namely 480G>C, has been described in the NCBI SNP database (January 2003), but its effect on folate concentrations has not yet been determined.

To examine the effect of the above-mentioned polymorphisms on folate metabolism in Czech population, we analyzed the relationship between five genetic variants (i.e. 1561C>T in FOLH1, 80G>A in RFC, 677C>T and 1298A>C in MTHFR, 480G>C in FOLR1 genes), and plasma or erythrocyte folate concentrations in 591 healthy Czech controls. Based on these analyses, we hypothesize on the possible effect of these genetic factors on folate status in the Czech Republic.

Methods

Subjects

For the analyses of genotypes and folates we used blood samples obtained from 591 healthy controls, who were recruited in a previously described study (Janošíková *et al.* 2003). The study exclusion criteria were as follows: age below 18 and above 65, domicile outside Central Bohemia or Prague, lactation, pregnancy, history of stroke or psychiatric disorders, presence of malignancies or of any symptoms of atherosclerosis. The history of smoking, consumption of beer and other alcoholic beverages, vegetables/fruit consumption, caloric restriction and vitamin use was obtained from a questionnaire. One hundred and sixty-two subjects used different multivitamins regularly, 80 individuals used multivitamins containing folates. The study was approved by the Ethics Committee of Charles University, First Faculty of Medicine; all subjects gave their written informed consent.

Laboratory analyses

Blood was obtained by standard venous puncture, using EDTA as anticoagulant. For determination of erythrocyte folates, 0.2 ml of blood were immediately mixed with 4 ml of 1 % ascorbic acid, left in dark at room temperature for 90 min and frozen at –20 °C prior to the analysis. Plasma was obtained by centrifugation at 2000 g for 15 min at 4 °C. Erythrocyte and plasma folates, vitamin B_{12} and B_6 (pyridoxal

5´phosphate) were determined by AxSYM Folate assay, AxSYM B_{12} assay (Abbott Laboratories, Abbott Park, Illinois) and Reagent kit for HPLC analysis of pyridoxal 5´phosphate in plasma/serum and whole blood (Chromsystems, München, Germany), respectively. Concentrations of aminothiols homocysteine (Hcy), cysteine (Cys), and glutathione (GSH) were determined by HPLC method using tris(2-carboxyl-ethyl) phosphine as a reductant of disulphide bonds and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate as fluorescent derivatization agent (Krijt *et al.* 2001).

Genomic DNA was isolated from whole blood using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). The methods of analysis for 677C>T and 1298A>C polymorphisms in methylentetrahydrofolate reductase gene by ARMS-PCR were published previously (Janošíková *et al.* 2003). The 80G>A in the reduced folate carrier gene was examined by PCR with allele specific primer pairs (forward primer, 5´-GGGAGGCCTGCAGACCATC-3´, reverse primer, 5´-AGCAAAGGTAGCACACGACGC-3´ for wild type allele, and 5´-AGCAAAGGTAGCACACGACGT-3´ for the variant allele, respectively). The polymorphism 1561C>T in folate hydrolase 1 gene was analyzed by the PCR-RFLP method as described previously (Devlin *et al.* 2000). The polymorphism 480 G>C in FOLR1 gene was detected by PCR-RFLP using a forward primer 5´-CCCCTGTGCAAAGAGGACTGT-3´ and a reverse primer 5´-CCCACTGCGCACTTGTTAAAC-3´. The 268 bp PCR product was digested by HpyCH4V, the presence of a control restriction site formed a 210 bp product from the normal allele, while the mutant allele was digested to 186 and 24 bp products. All PCR products were analyzed by electrophoresis in 3 % agarose gels stained with ethidium bromide. Detailed protocols can be requested from the authors.

Table 1. Aminothiol and vitamin concentrations in studied subjects

	Fasting concentrations			**Concentrations 6h after methionine load**		
	Folate non-users (n=511)	**Folate users (n=80)**	**p†**	**Folate non-users (n=511)**	**Folate users (n=80)**	**p†**
Plasma tHcy (μmol/l)	9.6 (8.2, 11.3)	9.0 (7.4, 10.8)	0.0249	35.4 (29.9, 43.1)	33.2 (27.6, 41.1)	0.0165
Plasma tCys (μmol/l)	299 (272, 328)	302 (282, 322)	0.4168	279 (252, 307)	279 (264, 296)	0.5634
Blood fGSH (μmol/l)	974 (759, 1197)	974 (827, 1149)	0.4841	995 (744, 1173)	1028 (856, 1132)	0.4574
Plasma folates (μmol/l)	14 (10.6, 17.7)	16.8 (12.8, 25.3)	<0.0001			
Erythrocyte folates (nmol/l)	716 (587, 907)	797 (703, 990)	0.0003			
Plasma vitamin B_6 (μg/l)	9.7 (6.6, 13.2)	11.4 (7,17.7)	0.0235			
Plasma vitamin B_{12} (pmol/l)	278 (215, 364)	286 (219, 338)	0.6650			

Data are presented as medians and 1st and 3rd quartile in parentheses. p†-value belongs to Mann-Whitney test for differences in medians between folate users and folate non-users populations. tHcy, total homocysteine, tCys, total cysteine, fGSH, non-protein bound (free) glutathione.

Statistical analyses

The proportions were tested using an exact binomial test, and continuous variables were compared by the Mann-Whitney non-parametric test. Plasma and blood folate levels were logarithmically transformed where appropriate, one subject with extremely low erythrocyte and normal plasma folate concentrations was excluded from selected statistical analyses due to a possible laboratory error (the sample was not available for repeated analyses). To assess the relationship between

the folate levels and gene variants we employed two different approaches: 1) linear regression of logarithm of folate levels with assumption of dose-response effect of variant allele, and 2) Mann-Whitney test comparing folate levels of subjects with at least one wild type allele to variant homozygotes. The first approach was complemented by multiple regression allowing for confounding variables (gender, age, smoking, consumption of beer and other alcoholic beverages, vegetables/fruit consumption, caloric restriction). All statistical analyses were performed at 5 % level of statistical significance using S+, version 6.0 and R, version 1.6.1.

Table 2. Characteristics of studied subjects

	Non- folate users	Folate users	All controls
Number of individuals	511	80	591
Males (%)	47	59	48
Age, median (years)	49 (42, 55)	51 (45.5, 54.25)	50 (42, 55)
WHR, median (m/m)	0.84 (0.80, 0.90)	0.87 (0.80, 0.90)	0.85 (0.80, 0.91)
BMI, median (kg/m^2)	25.9 (23.3, 28.3)	26.1 (23.8, 28.4)	25.9 (23.4, 28.4)
Smoking status, current+former (%)	43 (38, 47)	39 (28, 50)	42 (38, 46)
Smoking†, median (packyears)	4109 (1575, 7670)	5479 (2466, 9177)	4200 (1650, 7950)
Abstinents (%)	32 (28, 36)	24 (15, 35)	31 (27, 35)
Alcohol consumption‡, median (g/week)	84 (40, 187.5)	100 (55, 184)	95 (40, 187.5)
Hyperlipidemia (%)	24 (20, 28)	29 (19, 40)	24 (21, 28)
Hypertension (%)	13 (11, 17)	14 (7, 24)	13 (11, 16)
Diabetes mellitus (%)	4 (2, 6)	6 (2, 15)	4 (3, 6)

Data are presented as medians with 1st and 3rd quartile or proportions with 95 % CI in parentheses. Appropriate statistical tests were all non-significant at 5 % significance level. A diagnosis of hypertension, diabetes mellitus, or hyperlipidemia was defined as receiving current treatment for or having a past history of the condition. WHR, waist/hip ratio, BMI, body mass index, CAD, coronary artery disease, † current and former smokers only, ‡ non-abstinents only.

Table 3. Prevalence of genotypes and alleles in folate non-users and folate users population

Allelic variant	Folate non-users population (n=511)				Folate users (n=80)			
	Observed genotypes			f	Observed genotypes			f
	W/W	W/M	M/M	95 %CI	W/W	W/M	M/M	95 %CI
MTHFR c.677C>T A222V	211	249	51	0.34 (0.31-0.37)	34	38	8	0.34 (0.26-0.42)
MTHFR c.1298A>C E429E	226	235	50	0.33 (0.30-0.36)	38	33	9	0.32 (0.25-0.40)
RFC c.80G>A H27R	156	263	92	0.44 (0.41-0.47)	22	36	22	0.50 (0.42-0.58)
FOLH1 c.1561C>T H475Y	465	44	2	0.05 (0.03-0.06)	67	12	0	0.08 (0.04-0.13)
FOLR1 c.480G>C† W160C	50	0	0					

Data presented as numbers of individuals with the respective genotype (W, wild type allele, M, variant type allele), f, prevalence of the rare allele in the respective group with 95 %CI given in parentheses. †tested on 50 individuals (100 alleles) only; all alleles were in Hardy-Weinberg equilibrium in both the control set and the folate non-users subset. MTHFR, methylene tetrahydrofolate reductase, RFC, reduced folate carrier, FOLH1, folate hydrolase 1, FOLR1, folate receptor

Results

Of the 591 subjects, one fifth used various types of multivitamins regularly, only eighty individuals used multivitamins containing both folates and vitamin B_6 (for the latter group median daily intake of vitamins B_6, B_{12}, and of folate in multivitamin preparations was 2 mg, 6 μg and 400 μg, respectively). These 80 subjects taking folates and vitamin B_6 in multivitamins are further referred to as folate users. The folate users had significantly higher levels of plasma and erythrocyte folate, and of plasma vitamin B_6 than the folate non-users (16.8 nmol/l, 797 nmol/l and 11.4 μg/l vs. 14 nmol/l, 716 nmol/l and 9.7 μg/l, respectively). Congruently, the folate users showed lower plasma homocysteine levels.

The role of genetic variants of folate status was assessed in the subset of 511 folate non-users, who did not differ in demographic characteristics from the folate users (Table 2). The distribution of genotypes and variant allele frequencies in study group is presented in Table 3. The prevalence of the MTHFR 677C>T, MTHFR 1298A>C, FOLH1 1561C>T and RFC 80G>A variants in the Czech population was similar to that described in other Caucasians populations (Brattstrom *et al.* 1998, Devlin *et al*. 2000, Chango *et al.* 2000, Klerk *et al.* 2002, Van Der Put *et al.* 1998) and in Czechs (Beneš *et al.* 2001). The putative 480G>C variant in the FOLR1 gene was not found at any of the 296 examined control Czech chromosomes and was not further explored in the whole set.

Table 4. Plasma and blood folate levels in folate non-users population

Allelic variant	n	Observed genotypes			p linear trend	W/W +W/M	M/M	p Mann-Whitney
		W/W	W/M	M/M				
Median of plasma folate (nmol/l)								
MTHFR c.677C>T A222V	511	14.7	14.0	12.2	0.0422	14.3	12.2	0.0735
MTHFR c.1298A>C E429E	511	14.0	13.8	15.1	0.7542	13.9	15.1	0.2329
RFC c.80G>A H27R	511	14.8	13.8	13.4	0.4032	14.1	13.4	0.4916
FOLH1 c.1561C>T W160C	511	13.8	15.4	17.3	0.3780	13.8	15.4	0.256†
Median of blood folate (nmol/l)								
MTHFR c.677C>T A222V	510	710	700	876	0.0337	703	876	<0.0001
MTHFR c.1298A>C E429E	510	715	716	757	0.7079	716	757	0.9552
RFC c.80G>A H27R	510	711	734	690	0.5961	722	690	0.5225
FOLH1 c.1561C>T W160C	510	718	712	1019	0.6547	718	712	0.7012†

The relationship between plasma/blood folate levels and the four studied polymorphisms was assessed by testing for linear trend under the assumption of a dose-response effect of the studied alleles (p-values of unadjusted linear regression analyses are given in column p-linear trend) and by Mann-Whitney testing for comparison between the group of homozygotes for the variant allele and the group of heterozygotes pooled with the wild type homozygotes, assuming a recessive effect of the variant allele (p-values are given in column p-Mann-Whitney). W, wild-type allele, M, variant-type allele, n, number of individuals, † Only two homozygotes for the variant allele were detected, therefore the Mann-Whitney test was done for the group of wild type homozygotes and the pooled group containing heterozygotes and homozygotes for the variant allele.

To asses the relationship between plasma/blood folate levels and the four studied polymorphisms in the population of folate non-users, we employed two different statistical approaches: 1) a test for linear trend assuming a dose-response effect of the studied alleles, and 2) a test for comparison between the group of homozygotes for the variant allele and the group of heterozygotes pooled with the wild type homozygotes, assuming a recessive effect of the variant allele. The two methods agreed that there was no statistically significant effect of folate hydrolase 1, reduced folate carrier and MTHFR 1298A>C variants on plasma and blood folate levels (Table 4).

In contrast, the carriership of the MTHFR 677C>T variant was associated with a significant decrease of plasma folate levels. The median plasma folates in subjects with the CC, CT and TT genotypes were 14.7, 14.0 and 12.2 nmol/l, respectively, showing a significant linear trend with the increasing number of variant alleles on logarithmically transformed data suggesting a dose-response effect of the variant allele. The multiple linear regression analysis revealed four additional major determinants of folate status, namely gender, beer consumption, caloric restriction, and plasma levels of vitamin B_{12} (data not shown). However, their addition to the model did not attenuate the effect of the MTHFR 677C>T variant confirming that the MTHFR variant is indeed a significant independent determinant of folate status. In contrast to the plasma, the median erythrocyte folate levels were substantially higher in TT homozygotes than in the other two genotypes (867 vs. 703 nmol/l, respectively). In summary, our analyses of five SNPs show that only carriership of the 677C>T variant is associated with a significant effect on folate status in the Czech population, namely with decreased levels of plasma folate and with increased folate concentration in erythrocytes.

Discussion

In this study, we analyzed whether allelic variants in four genes of folate metabolism are determinants of plasma and erythrocyte folate levels in a homogenous Czech population. This population was especially suitable for analyzing the genetic component of folate status due to the absence of a national food fortification program and relatively low consumption of vitamin supplements compared to other populations (Ganji and Kafai 2003). Our findings in Czech subjects correspond to the previous publications (Ashfield-Watt *et al.* 2002, Van Der Put *et al.* 1998, 1995), which showed that TT homozygotes for MTHFR 677C>T polymorphism exibited lower plasma concentrations of folates than the wild type homozygotes.

Although the TT homozygosity was associated with decreased erythrocyte folate levels in many studies, several publications reported their increase (Nelen *et al.* 1998, Van Der Put *et al.* 1995). Our study yielded results similar to the latter reports as homozygotes for 677C>T polymorphism exhibited significantly higher erythrocyte concentration of folates. It was proposed that the incongruous effect of TT homozygosity on erythrocyte folate concentrations may result from different analytical method used in different published reports (Molloy *et al.* 1998, Ueland *et al.* 2001). Interestingly, increased erythrocyte folates were observed by us and by others if studies employed MEIA method with monoclonal antibodies to high-affinity specific folic acid binding protein (FABP). FABP has a greater capacity and stronger affinity for oxidized nonsubstitued folates (e.g.formylated forms) than for methyltetrahydrofolate (see Ax SYM folate assay kit manual, Abbott). As formylated folates are substantially increased in red blood cells of MTHFR 677TT homozygotes (Bagley and Selhub 1998), it is conceivable that the increased proportion of formylated erythrocyte folates in TT homozygotes may have lead to the overestimation of total erythrocyte folates by the MEIA method compared to other studies where the alternative method (HPLC, microbiological assays) was used.

For practical reasons, a crucial question should be answered: namely, whether the effect of MTHFR 677 T variant on plasma folates may be reversed by supplementing low doses of folic acid in diet or vitamins. Average dietary intake of folates in Czech population is 197 μg per day (Ruprich 1997), which is similar to that e.g. in the Netherlands (De Bree *et al.* 2003). Food fortification may increase the intake by 215-240 μg/day to approximately 400 μg per day (Quinlivan and Gregory 2003), a dose usually contained in multivitamins. Our cohort of 80 folate users contained only 8 homozygotes for the TT variant; owing to the low power we did not evaluate the effect of folates for individual genotypes. However, data from other studies demonstrated that carriers of mutant allele compared to CC individuals require higher dietary folate intake to achieve plasma folate levels comparable to CC individuals (De Bree *et al.* 2003). Taken together, the low dose of folic acid

supplements in multivitamins or in fortified food may not be sufficient for ~10 % of Caucasian individuals, namely for the TT homozygotes.

The other analyzed genetic variants (i.e. MTHFR 1298A>C, FOLH1 1561C>T and RFC 80G>A) were not significantly associated with blood or plasma folate levels, which corresponds to several previously published studies (Devlin *et al.* 2000, Fodinger *et al.* 2003, Chango *et al.* 2000). It is also important to note that the 1561C>T variant in the folate hydrolase 1 gene may have an effect on folate status, however, the low frequency of the variant allele requires larger samples to detect any significant association. In summary, it seems that except for the MTHFR 677C>T variant the other important genetic determinants of folate status, if any, have yet to be discovered.

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