Mutations in exons 2 and 3 of the *FOLR1* gene in demented and non-demented elderly subjects

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Abstract. We have previously reported six novel mutations in the 5'-UTR of the gene for folate receptor-α (*FOLR1*). In our search for additional mutations we screened patients, referred for investigation of suspected dementia (DGM subgroup) by SSCP and DNA sequencing from the end of exon 1 to the first bases of intron 3. We found 4 sequence variations, *FOLR1* g.1314G>A, g.1816delC, g.1841G>A, and g.1928C>T. Pyrosequencing™ genotyping assays were developed for all of them, and 389 active seniors (AS subgroup) and the 202 DGM patients were genotyped for these mutations. The frequency *q* of the mutated allele was, among the AS subjects, 0.068, 0.0026, 0.0026, and 0.024 respectively, and among the DGM subjects, 0.067, 0.0076, 0.0078, and 0.023. The g.1816delC and g.1841G>A mutations thus were more frequent in the DGM than in the AS subgroup, but the difference did not reach statistical significance. The mutated alleles, *FOLR1* 1816(-) and 1841A, always occurred together in the same subjects, suggestive of a rare double-mutant haplotype. The two common polymorphisms, *FOLR1* g. 1314G>A and g.1928C>T seemed not to raise tHcy plasma levels, whereas the double-mutated g.1816(-)-g.1841A haplotype may possibly have a slight tHcy-raising effect. Thus, so far 8 novel rare *FOLR1* mutations with a combined prevalence of ~1.3% in Whites as well as two common polymorphisms with 5% and 13%, respectively, have been demonstrated. Only a few of the rare mutations may potentially be associated with raised plasma tHcy concentrations. No association with dementia was found.

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Abbreviations: FR-α, folate receptor-α; RFC, reduced folate carrier; Hcy, homocysteine; MTHFR, methylenetetrahydrofolate reductase; *FOLR1*, folate receptor-α gene; TF, transcription factor; tHcy, plasma total homocysteine concentration; SSCP, single-strand conformation polymorphism; PCR, polymerase chain reaction; AD, Alzheimer's disease

Key words: folate receptor-α, homocysteine, *MTHFR*, pyrosequencing, SSCP, dementia

Introduction

Folate is important for all dividing cells. It is of utmost importance in providing methyl groups for remethylation of homocysteine (Hcy) to methionine and through S-adenosylmethionine (SAM) for one-carbon metabolism, providing cofactors for the synthesis of e.g. purines, phospholipids, and certain amino acids. Folate has been found to use several kinds of transport mechanism to enter the intracellular compartment. Reduced folate carrier (RFC) and receptor proteins are the most important ones. Folate receptor-α (FR-α) has high affinity for serum folate, 100-200 times higher affinity than RFC (1). Total plasma homocysteine (tHcy) is extensively used as a biomarker of shortage of e.g. folate. Elevated levels of tHcy can be due to mutations in enzymes involved in the Hcy metabolism, which cause dysfunctional enzymes, for instance the SNP at position 677 in the gene for MTHFR, methylenetetrahydrofolate reductase (2).

The gene for FR-α, named *FOLR1*, consists of seven exons and spans over 6.7 kbp (3). Mutations in the *FOLR1* gene could result in a dysfunctional receptor, or a lower amount of the receptor on the cell surface. The exons 3-7 in *FOLR1* are highly conserved and mutations have been considered to be very rare (4,5). However, a recent study reports novel mutations in the *FOLR1* exons 2 and 3 in Chinese patients (6), and we previously performed mutation screening in the 5'-UTR between nt. -1110 and nt. +272 and found six novel *FOLR1* mutations (7,8). The prevalence of the polymorphisms reported by Zhang *et al* in Europeans is not known. It is possible that the panorama of *FOLR1* polymorphisms or mutations differs between different parts of the world. It is also unclear whether they affect plasma tHcy concentrations.

Patients with dementia often have elevated tHcy concentrations, and studies have found a link between impaired Hcy metabolism and neuropsychiatric disorders such as depression (9) and cognitive impairment in the elderly (10). A lower amount of folate receptor-α expressed in cells could result in intra-cellular folate deficiency and consequently an elevated plasma concentration of tHcy. High concentrations of tHcy can induce DNA strand breakage, oxidative stress and apoptosis leading to neurodegenerative conditions (11). We therefore hypothesized that an increased prevalence of *FOLR1* mutations, or even additional dementia-specific mutations, might be found in subjects with a dementia diagnosis compared to healthy elderly subjects. This has not been studied before.

Table I. PCR primer sequences, annealing temperatures, and amplicon sizes for SSCP analysis of the *FOLR1* gene.

Amplicon	Primer sequence 5'-3'	Annealing temperature (˚C)	Size (bp)
nt. 1141 to nt. 1493	F: cctcctctcccaggtatgtgac R: cacctgcctagtctcaccttca	57	352
nt. 1419 to nt. 1781	F: ggtgtgggggtcatgattgt R: tgtggaatcatttcagcacaga	59	362
nt. 1723 to nt. 2054	F: ggtggtccctactgtgtgacttg R: gacctgctcactcactaacctt	61	332

Gene numbering according to GenBank nucleotide sequence ref. no. U20391.

Table II. PCR primer sequences, annealing temperatures, amplicon sizes and sequencing primer sequences for *FOLR1* genotyping by Pyrosequencing™ technology.

Polymorphism	dbSNP ID	Primer sequence 5'-3'	Annealing temperature (˚C)	Size (bp)	Sequencing primer 5'-3'
g.1314G>A	rs2071010	F: Biotin-tcccaccatgtgttaaggattt R: ggggatgaacactaacttgtcgt	59	139	tgaacactaacttgtcgttt
g.1816delC	rs3833748	F: tactgtgtgacttggggcatg R: Biotin-gcaaactatcttgaggcttagctt	56.5	135	tcatttgttgatttcccc
g.1841G>A	rs1540087	F: ggtggtccctactgtgtgactt R: Biotin-gcaaactatcttgaggcttagctt	60	144	ttacatttaatccttgcagga
g.1928C>T	rs9282688	F: taatccttgcaggagaaagctaagcc R: Biotin-cgggaacaaaccctaactgtttagag	64.5	140	tcgggacaggttgaa

Our aim was thus to analyse the frequency of the two mutations described by Zhang *et al* in Swedish elderly subjects, and to find out whether they have any effect on plasma tHcy concentrations. A second aim was to screen patients investigated for dementia for mutations in the same part of the *FOLR1* gene. For this purpose, we extended our search for additional mutations further downstream, spanning from the end of exon 1 to the first bases of intron 3.

Materials and methods

Subjects. We studied two groups of subjects: i) A group designated 'active seniors' (AS), which is a sample of 389 senior citizens, 262 women and 127 men, living in central Sweden. The subjects were retired and lived independently in their own homes. All were White and most of them were born in the 1920's and 1930's, the mean age at sampling was 74 years. All subjects gave a specific and written informed consent to the present study including genotyping and bio-banking of the donated samples. ii) The DGM study, which is a case control study at Örebro University Hospital, Sweden. This study population consisted of 202 consecutive patients (106 women and 96 men, mean age 72 years). They were all referred to the Memory Care Unit at the Department of Geriatrics for diagnostic assessment and treatment. Everyone in the study group underwent a structured clinical investigation, including medical history, family history and socioeconomic data, physical as well as neurological and psychiatric examination. Everyone was screened with mini mental state examination (MMSE) and clock drawing test (CDT). Computed tomography (CT) or magnetic resonance imaging (MRI) of the brain was performed on all but nine subjects. Dementia diagnoses were based on DSM-IV criteria. The ICD-10 criteria were used to divide patients into different diagnostic categories. Routine analyses of the Csf biomarkers, total tau protein, phosphorylated tau protein and ß-amyloid protein, used among the criteria for dementia diagostics, were performed at the Department of Psychiatry and Neurochemistry, Institute of Clinical Neuro-science, Sahlgrenska University Hospital, Mölndal, Sweden. Both studies were approved by the Research Ethics Committee of Örebro County Council.

Folate and Hcy measurements. Folate in serum was measured by a solid-phase time-resolved fluoroimmunoassay based on the competitive reaction between europium-labeled pteroylglutamic acid, the stable form of folate, and sample folate for a limited amount of binding sites on folate-binding protein (AutoDelfia™ Folate, Wallac Oy, Turku, Finland).

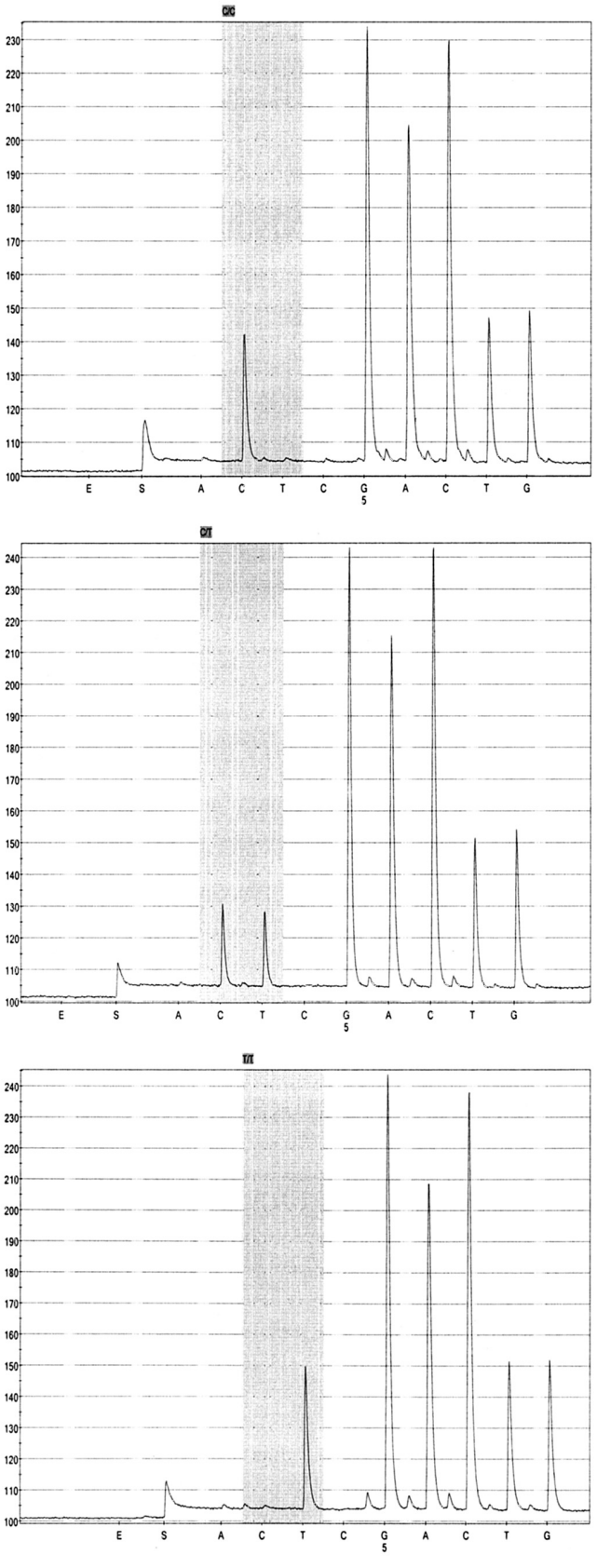

Figure 1. Typical pyrograms for the *FOLR1* g.1928C>T polymorphism. Genotypes shown, from top to bottom: CC, CT, TT.

Homocysteine in Stabilyte plasma was determined by a fluorescence polarization immunoassay on an IMx® unit (Abbott Laboratories, IL, USA). All coefficients of variation were <7.5%.

Mutation screening of FOLR1 by SSCP analysis and DNA sequencing. Genomic DNA was extracted from 200 μl whole blood using a QIAamp DNA blood mini kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). PCR was performed with the HotStarTaqDNA polymerase kit (Qiagen Inc.). Reaction mixture (50 μl) was used containing 0.4 μmol/l of each primer, 1.0 unit of Taq polymerase, 1.5 mmol/l $MgCl_2$, and 0.2 mmol/l each of dGTP, dATP, dTTP, and dCTP. Approximately 15 ng of the genomic DNA was added as a template. PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers, annealing temperatures, and amplicon sizes are shown in Table I. The 5'-UTR-region of the folate receptor-α gene was searched for mutations by single-strand conformation polymorphism (SSCP) screening using preformed gels and GeneGel SSCP buffer kits A, B, and C, and a silver staining kit (all from Amersham Biosciences, Uppsala, Sweden) and DNA sequencing using Big Dye Terminator reagents as described previously (7). The first studied 352-bp amplicon stretched from nt. 1141 to nt. 1493 according to Genbank sequence U20391. The second studied amplicon stretched from nt. 1419 to nt. 1781 and the third studied amplicon from nt. 1723 to nt. 2054.

Development of FOLR1 SNP assays by Pyrosequencing™. The polymorphisms in the *FOLR1* gene that had been identified by SSCP screening were further tested for, in active seniors and in the dementia study population, by SNP assays on the Pyrosequencing technology platform. The four SNPs were analysed by newly developed Pyrosequencing protocols. The primers were designed using the PSQ assay design software, version 1.0.6 (Biotage AB, Uppsala, Sweden). The PCR primer and sequence primer data and annealing temperatures are summarized in Table II. After PCR the samples were prepared using the Vacuum Prep workstation (Biotage AB): 25-30 μl of the amplicon, 3 μl Streptavidin Sepharose HP beads (Amersham Biosciences), 37 μl binding buffer [10 mM Tris-HCl, 2M NaCl, 1 mM EDTA, 0.1% Tween-20, Milli-Q (18.2 MΩ x cm) water, pH 7.6] and 10-15 μl Milli-Q water were mixed and used in the Vacuum Prep workstation. The biotinylated amplicons were immobilized onto the streptavidin sepharose beads and then passed through three washing steps using the Vacuum Prep workstation. The first wash was 70% ethanol, the second was 0.2 M NaOH, and the third washing buffer was 10 mM Tris-Acetate and Milli-Q water. The amplicons were then transferred to a plate containing sequencing primer (0.4 μM) in 40 μl annealing buffer (20 mM Tris-Acetate, 2 mM magnesium acetate, pH 7.6). The amplicons and sequencing primer were incubated at 80˚C for 2 min and then left to cool at room temperature to allow annealing of the sequencing primer. Sequencing was performed using a PSQ96 SNP reagent kit and a PSQ 96MA system (Biotage AB). The nucleotide addition order was optimised by the PSQTM96MA 2.0.1 software. Results were automatically analysed using the PSQ96MA 2.0.2 software. Figs. 1-4 show the pyrograms obtained for each of the three possible genotypes at the *FOLR1* g.1928C>T, g.1314G>A, g.1816delC, and 1841G>A loci, respectively.

MTHFR 677C>T was amplified according to the Pyrosequencing assay protocol 'Genotyping of the C677T variant

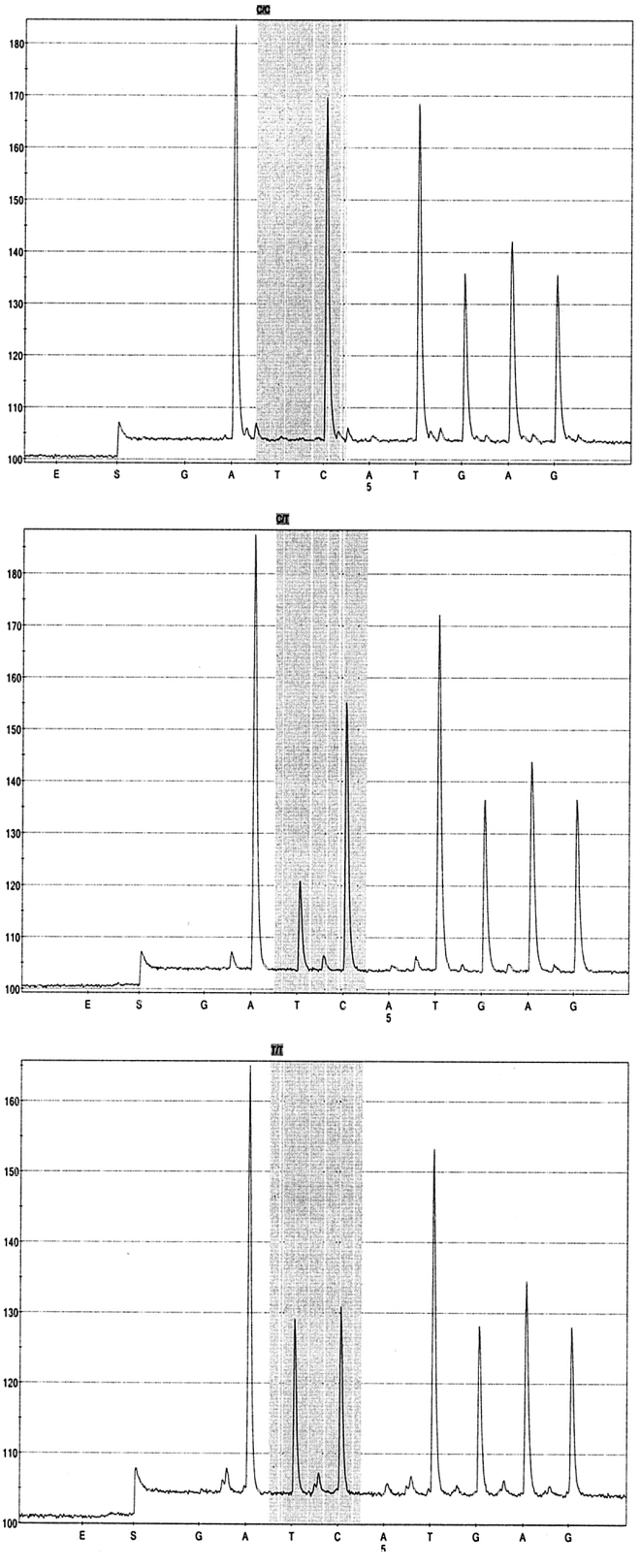

Figure 2. Typical pyrograms for the *FOLR1* g.1314G>A polymorphism. Genotypes shown, from top to bottom: GG, GA, AA. This is a reverse assay, so the pyrogram reading CC corresponds to the genotype GG, CT to GA, TT to AA. The other three novel assays presented here are forward assays where the genotypes are read directly from the output format.

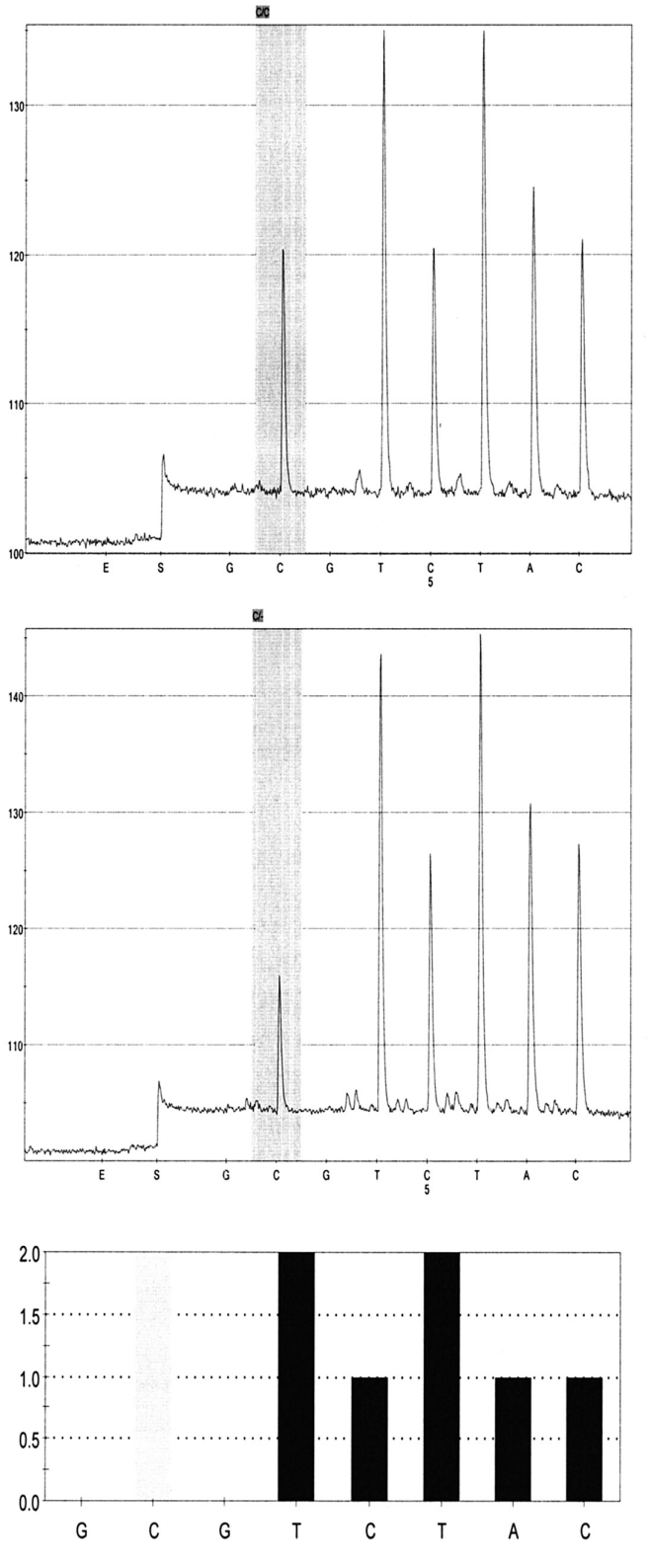

Figure 3. Typical pyrograms for the *FOLR1* g.1816delC polymorphism. Genotypes shown, from top to bottom: CC, C(-). The third possible genotype (-/-) was not found in the studied population. However, if present it would be easily distinguishable since the first C-peak, which in the heterozygote is reduced to half-height (lower panel), would disappear completely in a homozygous subject (lower panel).

in the human methylenetetrahydrofolate reductase (MTHFR) gene', version 1, from Biotage AB, Uppsala, Sweden (www.biotage.com). Approximately 60 ng of genomic DNA was used as a template.

Results

Mutation screening of FOLR1 in DGM patients. We screened the DGM patients for mutations using SSCP, followed by DNA

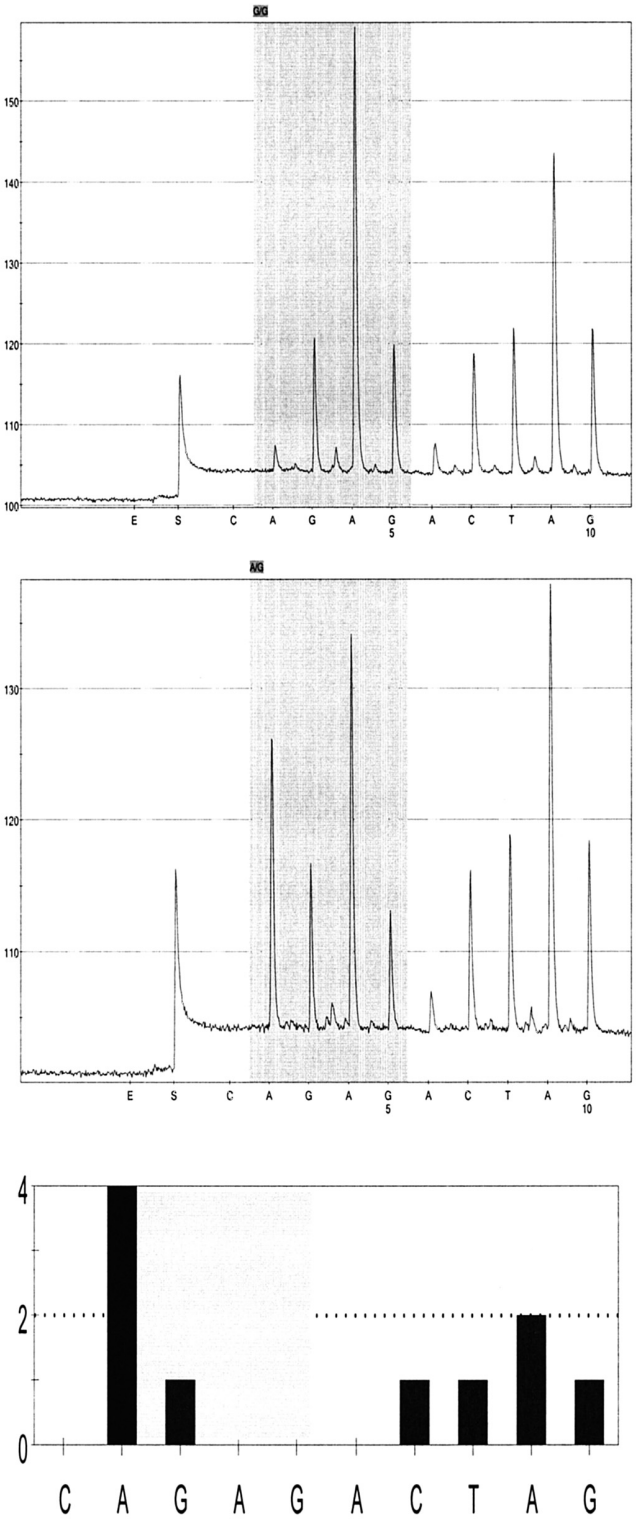

Figure 4. Typical pyrograms for the *FOLR1* g.1841G>A polymorphism. Genotypes shown, from top to bottom: GG, GA. The third possible genotype (AA) was not found in the studied population. However, if present it would be easily distinguishable since it would differ from the other two genotypes with respect to three different positions in the pyrogram (lower panel).

sequencing of any samples showing a band pattern indicative of mutations. Fig. 5 shows DNA sequencing chromatograms of each of the four identified mutations. We followed the nomenclature by den Dunnen and Antonarakis (12) and chose the U20391 as the reference sequence. We also referred to the exon/intron structure as given by Elwood *et al* (3).

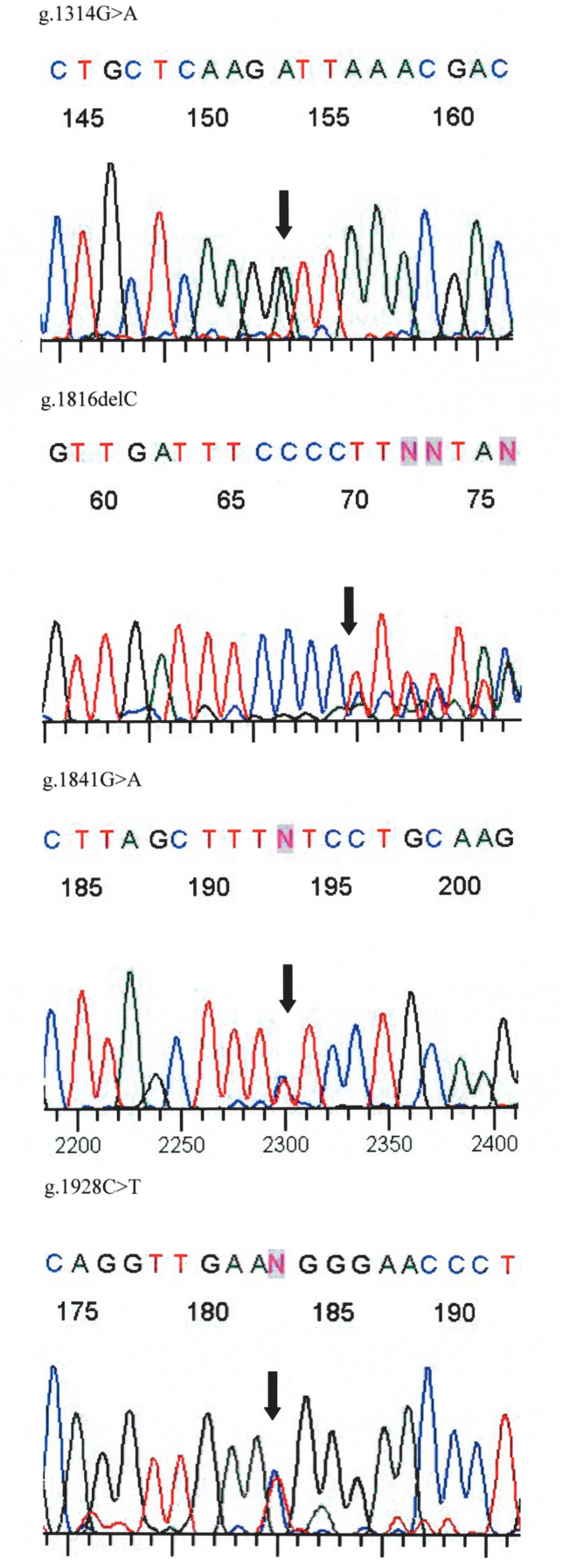

Figure 5. DNA sequencing chromatograms of the four *FOLR1* mutations described. All chromatograms show the forward sequence. Arrows indicate the position of the mutation. From top to bottom: g.1314G>A, g.1816delC (the sequence turns out of phase following the deletion), g.1841G>A, and g.1928C>T.

Table III. Number of subjects with *FOLR1* mutations among active seniors and DGM patients.[a]

	Active seniors			DGM patients		
Genotype/allele	Frequency	n	χ^2	Frequency	n	χ^2
g.1314G>A						
G/G	0.869	338		0.872	168	
G/A	0.126	49		0.122	24	
A/A	0.005	2	0.024	0.005	1	0.020
p(G)	0.932			0.932		
q(A)	0.068			0.067		
g.1816delC						
C/C	0.995	387		0.984	190	
C/-	0.005	2		0.016	3	
-/-		0	0.0026		0	0.0118
p(C)	0.997			0.992		
q(-)	0.0026			0.0078		
g.1841G>A						
G/G	0.995	386		0.984	189	
G/A	0.005	2		0.016	3	
A/A		0	0.0026		0	0.0119
p(G)	0.997			0.992		
q(A)	0.0026			0.0078		
g.1928C>T						
C/C	0.951	368		0.953	184	
C/T	0.049	19		0.047	9	
T/T		0	0.245		0	0.110
p(C)	0.975			0.977		
q(T)	0.024			0.023		

[a]Allele frequencies and χ^2 for Hardy-Weinberg equilibrium are shown. Data are based on genotyping performed by the novel Pyroseqencing assays (see Materials and methods).

In the amplicon spanning nt. 1141 to nt. 1493, immediately downstream and partly overlapping the region which we screened in our prior study on hyperhomocysteinemic patients (8), we found a G>A mutation (Fig. 5, top panel), and designated it *FOLR1* g.1314G>A. It was identical to the mutation designated 1314A>G in a previous study by Zhang *et al* (6).

In the amplicon spanning nt. 1419 to nt. 1781 we did not find any mutations.

In the amplicon spanning nt. 1723 to nt. 2054 we found three mutations (Fig. 5, three last panels), designated Ex3+205C>- , Ex3-195G>A and Ex3-108C>T by the Cancer Genome Anatomy Project SNP500Cancer database (13). Ex3+205C>- in this database is identical to the mutation designated C1816delC in the study by Zhang *et al* (6). We designated these three mutations *FOLR1* g.1816delC, *FOLR1* g.1841G>A, and *FOLR1* g.1928C>T respectively. Our designations follow guidelines by den Dunnen and Antonarakis (12). The updates posted on http://www.hgvs.org/mutnomen/recs-DNA.html have also been consulted.

Frequency of FOLR1 mutations. The frequency of the mutations identified by SSCP screening was studied among active senior subjects and DGM patients. Testing was performed by Pyrosequencing technology as described in Materials and methods (Figs. 1-4). The allele frequencies found in these two clinical groups are shown in Table III. As seen from the χ^2 figures, all mutations were in Hardy-Weinberg equilibrium. Of note was the finding that the 5 subjects who were heterozygous for the g.1816delC mutation were all heterozygous also for the g.1841G>A mutation. No subject was found to be a carrier only of one of these two mutations (Table IV). The most likely explanation for this

Table IV. Plasma levels of tHcy and serum folate and B12 levels in subjects with *FOLR1* mutations g.1816delC and g.1841G>A.

Subject	Diagnosis	tHcy, μmol/l	S-folate, nmol/l	B12, pmol/l	g.1816delC	g.1841G>A	MTHFR 677C>T
1	ND	7.8	19.7	339	C/-	GA	CC
2	ND	10.0	13.1	269	C/-	GA	CC
3	AD	17.0	7.3	367	C/-	GA	CC
4	ND	17.0	12.1	396	C/-	GA	CT
5	AD	29.0	8.0	233	C/-	GA	CT

Individual values are shown. The MTHFR 677C>T status of each subject is also indicated. Diagnosis is provided for subjects from the dementia study cohort. AD, Alzheimer's disease; ND, non-dementia. Subjects 1 and 2 were from the AS cohort.

Table V. Plasma levels of tHcy and serum folate in subjects with FOLR1 mutation g.1314G>A.

1314G>A	n	tHcy, μmol/l		P (Anova)	n	S-folate, nmol/l		P (Anova)
Genotype		Median	Mean ± SD			Median	Mean ± SD	
Subjects with S-folate ≥20 nmol/l were excluded								
GG	370	13.00	13.87±4.75	0.182	371	10.30	10.84±3.51	0.532
GA	56	13.00	13.78±4.71		57	9.60	10.28±3.51	
AA	3	20.39	18.93±3.08		3	11.40	10.97±2.18	
Subjects with S-folate ≥20 nmol/l and subjects with the MTHFR 677TT genotype were excluded								
GG	342	13.00	13.85±4.78	0.123	343	10.30	10.85±3.47	0.602
GA	53	13.00	13.67±4.66		55	9.60	10.42±3.53	
AA	2	20.70	20.70±0.43		2	12.15	12.15±1.06	
Subjects with S-folate ≥20 nmol/l and subjects with the MTHFR 677TT genotype or subjects with the 1928 CT genotype were excluded								
GG	316	13.00	13.93±4.85	0.134	317	10.20	10.81±3.48	0.461
GA	51	13.00	13.72±4.66		52	9.45	10.24±3.42	
AA	2	20.70	20.70±0.43		2	12.15	12.15±1.06	

Median, mean, and SD values are shown.

would be that, in our population, the mutated alleles occurred together in a double-mutated haplotype, 1816(-)-1841A with a mean frequency of approximately q=0.0043 for the combined study groups. The frequencies of the mutated alleles were higher in the DGM than in the AS study group, but the differences were not statistically significant, χ^2=1.62 and 1.65, respectively.

Hcy levels in subjects with FOLR1 mutations. Table IV shows the tHcy levels and related variables in all subjects studied who carried the double-mutant haplotype 1816(-)-1841A. Subjects 1 and 2 had satisfactory S-folate levels and consequently low tHcy levels. Subjects 3 and 5 had low to normal S-folate levels (reference interval in our routine laboratory 8-43 nmol/l) and tHcy levels higher than expected from their S-folate levels. Subject 4 had satisfactory S-folate levels but slightly raised tHcy levels. None of the subjects had the MTHFR 677TT genotype, but subjects 4 and 5 had 677 CT heterozygosity.

The mutations g.1314G>A and g.1928C>T were much more prevalent and are clearly polymorphisms. The possible effect of these on the tHcy/folate interplay was therefore studied by regression analysis. Fig. 6 shows the relation between serum folate levels and plasma tHcy concentration in subjects with or without the g.1314G>A mutation, respectively. Subjects with S-folate ≥20 nmol/l, subjects with the MTHFR 677TT genotype, and subjects carrying the mutated *FOLR1* g.1928T allele were excluded from the analysis. In 1314GG (wild-type) subjects, the regression showed a significant negative slope, whereas the slope did not differ significantly from zero in the 1314A-allele carriers. Table V shows mean tHcy and S-folate levels according to 1314 genotype, before and after exclusion of subjects with MTHFR 677TT and 1928T-allele carriers, respectively. The 1314A-allele does

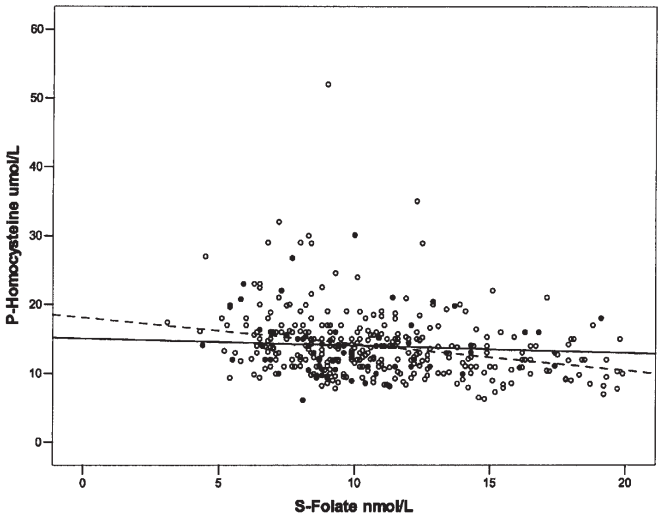

Figure 6. Scatter plot of tHcy and S-folate values in subjects with the *FOLR1* g.1314GG genotype (○) and with the *FOLR1* g.1314GA or AA genotype (●). Only subjects with an S-folate of <20 nmol/l were included in the figure. Subjects with the MTHFR 677TT and the FOLR1 g.1928CT genotypes were also excluded. The regression equations were for the GG-genotype: tHcy = -0.38 (-0.5; -0.2) x S-folate + 18.1 (16.4; 19.8) and for the combined GA and AA-genotypes: tHcy = -0.10 (-0.5; 0.3) x S-folate + 15.1 (10.9; 19.3). Parentheses show the $P_{2.5}$ and $P_{97.5}$ of the parameters (i.e., the 95% CI).

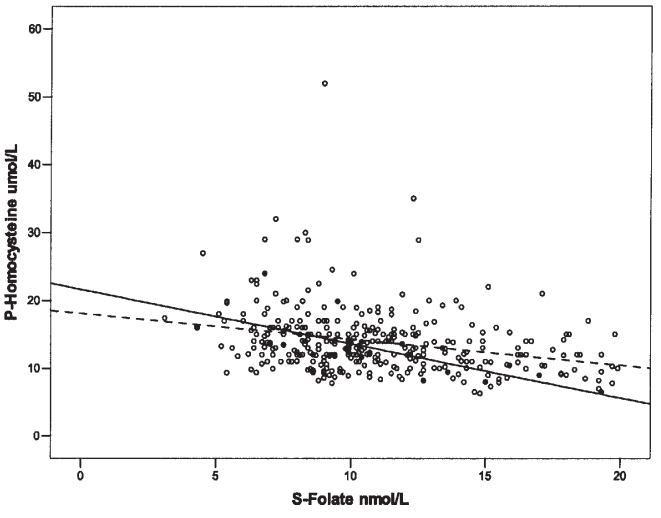

Figure 7. Scatter plot of tHcy and S-folate values in subjects with the *FOLR1* g.1928 CC genotype (○) or with the 1928CT genotype (●). Only subjects with an S-folate of <20 nmol/l were included in the figures. Subjects with the MTHFR 677TT and the *FOLR1* g.1314GA or AA genotypes were also excluded. The regression equations were for the CC-genotype: tHcy = -0.38 (-0.5; -0.2) x S-folate + 18.1 (16.4; 19.8) and for the CT-genotype: tHcy = -0.80 (-1.2; -0.4) x S-folate + 21.6 (16.6; 26.7). Parentheses show the $P_{2.5}$ and $P_{97.5}$ of the parameters (i.e., the 95% CI).

Table VI. Plasma levels of tHcy and serum folate in subjects with *FOLR1* mutation g.1928C>T.

1928C>T	n	tHcy, μmol/l		P (Anova)	n	S-folate, nmol/l		P (Anova)
Genotype		Median	Mean ± SD			Median	Mean ± SD	
Subjects with S-folate ≥20 nmol/l were excluded								
CC	405	13.00	13.97±4.78	0.165	407	10.20	10.69±3.48	0.050
CT	22	12.95	12.53±4.27		22	12.10	12.20±3.86	
Subjects with S-folate ≥20 nmol/l and subjects with the MTHFR 677 TT genotype were excluded								
CC	374	13.00	13.92±4.81	0.300	376	10.20	10.74±3.46	0.108
CT	21	13.00	12.81±4.16		21	11.90	11.99±3.82	
Subjects with S-folate ≥20 nmol/l and subjects with the MTHFR 677 TT genotype or with the 1314GA or AA genotype were excluded								
CC	316	13.00	13.93±4.85	0.425	317	10.20	10.81±3.48	0.277
CT	20	13.25	13.05±4.12		20	11.75	11.69±3.66	

Median, mean, and SD values are shown.

not seem to be associated with increased plasma tHcy concentrations.

Fig. 7 shows the relation between serum folate levels and plasma tHcy concentrations in subjects with or without the g.1928C>T mutation, respectively. Subjects with S-folate ≥20 nmol/l, subjects with the MTHFR 677TT genotype, as well as subjects carrying the *FOLR1* g.1314A-allele (genotypes GA and AA) were excluded. There was no statistically significant difference between the regression equations for the subjects wild-type for the *FOLR1* g.1928C>T or the subjects which were carrying the *FOLR1* g.1928T-allele. Table VI shows mean tHcy and S-folate levels according to 1928C>T genotype, before and after exclusion of subjects with folate ≥20 nmol/l, MTHFR 677TT subjects, and 1314A-allele carriers respectively. The 1928T-allele does not seem to be associated with increased plasma tHcy concentrations.

Discussion

We here present an extension of our mutational screening of the *FOLR1* gene (7,8) to approximately 1 kb further downstream in the sequence, in which we found 4 mutations. We

also developed Pyrosequencing™ genotyping assays for these mutations. Two of them turned out to be the same as those described in a recent study by Zhang *et al* (6) and three were contained in the SNP500Cancer database (13). Very scarce data, if any, is available on the allele frequencies of these 4 mutations in Whites, and no data on relations with tHcy or folate levels have been published. We used our novel Pyrosequencing assays to determine the allele frequencies in our biobanks of elderly Swedes.

For the *FOLR1* g.1841G>A mutation the allele frequency of the A-allele reported in the NCBI SNP database, dbSNP ID rs1540087, is 0.00 in an unspecified Caucasian population. According to this NCBI SNP database entry the A-allele appears to be most frequent in Africans with a frequency of 0.043. However, we found the 1841A-allele frequency in elderly Swedish subjects to be readily detectable (q=0.0026 in the AS subgroup and q=0.0078 in the DGM subgroup).

For the *FOLR1* g.1928C>T polymorphism we found the T-allele frequency in the AS subgroup to be 0.024 and in the DGM subgroup to be 0.023. The reported allele frequency for the T-allele in Caucasians is 0.054 in the NCBI SNP database. The highest among the populations recorded are Africans, q(T)=0.021; Hispanic, q(T)=0.022; and Pacific Rim heritage, q(T)=0.000. Thus, Swedes, Africans, and Hispanics actually have very similar allele frequencies, and the NCBI SNP database frequency for unspecified 'Caucasians' may not be representative of Whites in general.

The two rather high-frequency polymorphims described by Zhang *et al* (6), g.1314G>A and 1816delC, were present also in the Swedish population, both in demented subjects and in active senior subjects (Table III). The frequency of the 1314A-allele was appreciably lower than in China, q being only 0.068 (AS subgroup) and 0.067 (DGM subgroup) vs. 0.186 (cancer cases) and 0.143 (controls) in the Chinese. Likewise q of the g.1816delC polymorphism was only 0.0026 (AS subgroup) and 0.0078 (DGM subgroup) in the Swedes vs. 0.095 (cancer cases) and 0.099 (controls) in the Chinese. For the *FOLR1* g.1314G>A mutation the reported allele frequency q(A), in the NCBI SNP database, was 0.104 in a European population and for *FOLR1* g.1816delC the mutated allele frequency q(-) was 0.034 in a Caucasian population. Our frequencies of these two mutations are thus lower than those reported both in a Chinese, and, according to the NCBI database, in an unspecified European population.

We here provide the first frequency estimate of the *FOLR1* g.1816delC mutation, q=0.0026 in Swedish senior citizens and q=0.0078 in patients referred for investigation of suspected dementia. We also found that this mutation never occurred alone but only in the simultaneous presence of the mutated *FOLR1* g.1841A allele, suggestive of a double-mutated haplotype 1816(-)-1841A. The finding needs corroboration by molecular haplotyping and in larger population samples. For instance, it would be very interesting to know the frequency of the *FOLR1* g.1841A-allele among the Chinese, and whether it occurs in complete linkage with the g.1816delC mutation in the Chinese too. The possibility of a difference in frequency of the *FOLR1* 1816(-)-1841A haplotype between active seniors and demented subjects, raised by the present findings, must await confirmation from larger studies, which it would be advantageous to conduct in countries with a higher frequency of these two mutations. On the other hand, the 1314G>A and the 1928C>T polymorphisms clearly did not differ between the AS and the DGM groups.

Do any of the 4 novel *FOLR1* mutations raise plasma tHcy concentrations and/or lower serum folate concentrations? One needs to integrate all relevant available nutrigenetic backgound data of each subject in order to answer that question. We adjusted for both the MTHFR 677C>T genotype and for the novel *FOLR1* mutations and S-folate in the present study, and precluded any major impact on tHcy or folate levels of the 1314G>A and 1928C>T polymorphisms (Figs. 6 and 7, Tables V and VI), whereas the double-mutated *FOLR1* 1816(-)-1841A haplotype may possibly be associated with higher plasma tHcy levels although 3 out of 5 studied subjects were inconclusive for this purpose due to their MTHFR genotypes and/or S-folate levels (Table IV).

In conclusion, we here describe 4 additional *FOLR1* mutations, their allele frequencies and relations to total plasma homocysteine. This brings the number of recently discovered mutations in this gene and its proximal 5' upstream sequence up to 10. The combined prevalence of heterozygosity for the eight low-frequency mutations in Swedes is approximately 1.3-1.5% and there are two common polymorphisms, g.1314>A and g.1928C>T with approximately 13% and 5% heterozygotes, respectively, in the Swedish population. While the two latter seem not to raise tHcy plasma levels, the double-mutated *FOLR1* g.1816(-)-g.1841A haplotype may possibly have a tHcy-raising effect. Its frequency, as well as its possible effect on tHcy, should be studied in additional populations in order to gain a fuller picture of both the history and the effects of this interesting haplotype.

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References

1. Kamen BA and Smith AK: A review of folate receptor alpha cycling and 5-methyltetrahydrofolate accumulation with an emphasis on cell models *in vitro*. Adv Drug Deliv Rev 56: 1085-1097, 2004.
2. Frosst P, Blom HJ, Milos R, *et al*: A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 10: 111-113, 1995.
3. Elwood PC, Nachmanoff K, Saikawa Y, *et al*: The divergent 5' termini of the alpha human folate receptor (hFR) mRNAs originate from two tissue-specific promoters and alternative splicing: characterization of the alpha hFR gene structure. Biochemistry 36: 1467-1478, 1997.
4. Heil SG, van der Put NM, Trijbels FJ, Gabreels FJ and Blom HJ: Molecular genetic analysis of human folate receptors in neural tube defects. Eur J Hum Genet 7: 393-396, 1999.
5. Barber RC, Shaw GM, Lammer EJ, *et al*: Lack of association between mutations in the folate receptor-alpha gene and spina bifida. Am J Med Genet 76: 310-317, 1998.
6. Zhang G, Zhang QY, Miao XP, Lin DX and Lu YY: Polymorphisms and mutations of the folate receptor-alpha gene and risk of gastric cancer in a Chinese population. Int J Mol Med 15: 627-632, 2005.
7. Nilsson TK and Börjel AK: Novel insertion and deletion mutations in the 5'-UTR of the folate receptor-alpha gene: an additional contributor to hyperhomocysteinemia? Clin Biochem 37: 224-229, 2004.

8. Börjel AK, Yngve A, Sjöström M and Nilsson TK: Novel mutations in the 5'-UTR of the FOLR1 gene. Clin Chem Lab Med 44: 161-167, 2006.
9. Bottiglieri T, Laundy M, Crellin R, Toone BK, Carney MW and Reynolds EH: Homocysteine, folate, methylation, and monoamine metabolism in depression. J Neurol Neurosurg Psychiatry 69: 228-232, 2000.
10. Seshadri S, Beiser A, Selhub J, *et al*: Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 346: 476-483, 2002.
11. Mattson MP and Shea TB: Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders. Trends Neurosci 26: 137-146, 2003.
12. den Dunnen JT and Antonarakis SE: Mutation nomenclature extensions and suggestions to describe complex mutation: a discussion. Hum Mutat 15: 7-12, 2000.
13. Packer BR, Yeager M, Burdett L, *et al*: SNP500Cancer: a public resource for sequence validation, assay development, and frequency analysis for genetic variation in candidate genes. Nucleic Acids Res 34: D617-D621, 2006.
14. Wolters M, Hermann S and Hahn A: Effect of multivitamin supplementation on the homocysteine and methylmalonic acid blood concentrations in women over the age of 60 years. Eur J Nutr 44: 183-192, 2005.