European Journal of Human Genetics (2007) 15, 103–109

www.nature.com/ejhg

ARTICLE

Molecular genetic analysis of the human dihydrofolate reductase gene: relation with plasma total homocysteine, serum and red blood cell folate levels

Henkjan Gellekink[1,2], Henk J Blom[1], IJM van der Linden[1] and Martin den Heijer*[,2,3]

[1]*Laboratory of Paediatrics and Neurology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands;*
[2]*Department of Endocrinology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands;*
[3]*Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands*

Disturbances in folate metabolism may increase the risk of certain malignancies, congenital defects and cardiovascular diseases. The gene dihydrofolate reductase (*DHFR*) is primarily involved in the reduction of dihydrofolate, generated during thymidylate synthesis, to tetrahydrofolate in order to maintain adequate amounts of folate for DNA synthesis and homocysteine remethylation. In order to reveal possible variation that may affect plasma total homocysteine (tHcy), serum folate and red blood cell (RBC) folate levels, we sequenced the *DHFR* coding region as well as the intron–exon boundaries and *DHFR* flanking regions from 20 Caucasian individuals. We identified a 9-bp repeat in the 5′-upstream region that partially overlapped with the 5′-untranslated region, and several single-nucleotide polymorphisms, all in non-coding regions. We screened subjects for the 9-bp repeat ($n=417$), as well as the recently reported 19-bp deletion in intron 1 ($n=330$), and assessed their associations with plasma tHcy, serum and RBC folate levels. The 19-bp del/del genotype was associated with a lower plasma tHcy (−14.4% [95% confidence interval: −23.5 to −4.5], $P=0.006$) compared with the wild-type genotype. This may suggest that intracellular folate levels are affected.
European Journal of Human Genetics (2007) **15,** 103–109. doi:10.1038/sj.ejhg.5201713; published online 13 September 2006

Keywords: folate; dihydrofolate reductase; genetic variation; homocysteine

Introduction

Folate, a water-soluble B-vitamin, has gained increasing interest for its essential role in DNA synthesis/repair and the transmethylation pathway. The primary function of folate is as a carrier of one-carbon units for purine and thymidine synthesis and the conversion of homocysteine to methionine. Because of the close interrelation between folate and homocysteine, low folate status is reflected by elevated plasma total homocysteine (tHcy) concentrations.[1,2] A number of diseases have been linked to disturbances in the above-mentioned pathways. For example, low folate status increases the risk of certain cancers[3–5] and is related to congenital malformations, such as neural tube defects.[6] Moreover, an increase in tHcy increases the risk of cardiovascular disease and congenital abnormalities.[7–9]

Dietary folates, present in reduced and partially reduced form,[10] exist as polyglutamates and require hydrolysis to

*Correspondence: Dr M den Heijer, Department of Endocrinology (471), Radboud University Nijmegen Medical Centre, PO Box 9101, Nijmegen 6500 HB, The Netherlands.
Tel: +31 24 3614599; Fax: +31 24 3618809;
E-mail: m.denheijer@endo.umcn.nl
Received 19 April 2006; revised 26 June 2006; accepted 19 July 2006; published online 13 September 2006

monoglutamates before absorption in the intestine. After release into the portal circulation, folate (mainly as 5-methyltetrahydrofolate) is distributed throughout the body and is used for homocysteine remethylation, leaving tetrahydrofolate (THF) and methionine. Methionine is used for protein synthesis or converted to *S*-adenosylmethionine, the principle methyl donor in the human body. THF is converted to methyleneTHF, among others, and used for thymidylate synthesis. The latter generates dihydrofolate (DHF) that requires the action of dihydrofolate reductase (DHFR) to be reduced to THF again. In addition, folic acid present in vitamin pills and fortified foods requires full reduction by DHFR via DHF into THF in order to become metabolic active. This makes DHFR an essential enzyme not only for the initial reduction of dietary folates (and folic acid) but also for the continuous reduction of DHF into THF generated during thymidylate synthesis.[11]

DHFR (EC 1.5.1.3) is located on chromosome 5q11.2-q13.2 and spans about 30 kb. The gene consists of six exons, yielding an mRNA of about 3.9 kb.[11] DHFR enzyme (AC 008434.5) is a cytosolic protein of 21.3 kDa and requires NADP as a cofactor. At least three pseudo genes of *DHFR* are known, that is, *DHFRP1* (chromosome 18), *DHFRP2* (chromosome 6) and *DHFRP4* (chromosome 3, also denoted *DHFRL1*),[12,13] which are highly homologous to the corresponding *DHFR* coding regions. Outside these regions, sequence homology is relatively low.[14] DHFR deficiency (OMIM 126060) is sporadically seen and results in severe megaloblastic anemia and mild to moderate mental retardation.[15,16] The antifolate methotrexate (MTX) is well known for its inhibitory effect on DHFR thereby preventing DNA synthesis and, hence, tumor growth.[17] In addition, inhibition of DHFR by MTX and the antibiotic trimethoprim were shown to increase tHcy levels by 50%,[18,19] suggesting that DHFR dysfunction owing to genetic variants may affect tHcy as well. Recently, Johnson *et al*.[20] described a 19-bp deletion that may affect gene expression.

In this study, we aimed to identify genetic variation within the *DHFR* gene by sequencing the entire coding region, including the intron–exon boundaries and *DHFR* flanking regions. Newly identified variants, and the 19-bp deletion in intron 1,[20] were assessed for their effect on tHcy and serum and red blood cell (RBC) folate concentrations in population-based controls.

Materials and methods

Study population

DNA from 20 individuals of Caucasian origin, who had participated in an earlier study,[21] were used for *DHFR* sequence analysis. Functional polymorphisms in the *DHFR* gene are likely to be reflected in plasma folate and homocysteine levels. The subjects were selected based on these criteria: six subjects had low folate (<10th percentile = 7.0 nmol/l) and high homocysteine levels (>90th percentile = 15.5 μmol/l), nine subjects had a plasma tHcy above 15.5 μmol/l and five subjects were selected randomly. Subjects with low vitamin B12 (<10th percentile = 124.7 pmol/l) or the *MTHFR* 677TT genotype were excluded. We aimed to identify common genetic variants that may contribute to changes in tHcy or folate at the population level. According to a binomial distribution, the chance of finding an allele with a frequency of 2.5% in one out of 40 alleles is 0.64. In addition, the minimum allele frequency required to find at least one out of 40 alleles (with 95% confidence) is 7.2%. The population used for the association study was recruited via a general practice in The Hague (The Netherlands) and has been described in more detail elsewhere.[21] In total, 2812 people, 20–90 years of age, were invited to take part in a health survey on risk factors for cardiovascular disease. From the group that agreed to participate ($n = 532$), the first 500 were included. For the current study, DNA was available of 438 individuals. All participants gave their informed consent.

Sequence analysis of the *DHFR* gene

Blood samples were drawn from the antecubital vein. DNA was extracted,[22] and then stored at 4°C. Genomic DNA sequencing of the entire coding region (GenBank accession number NT_006713, AC008434.5) was performed in order to screen the *DHFR* gene for variation. We also sequenced 665 bp of the 5′-upstream region, which included the 5′-untranslated region (UTR) of about 500 bp (NM_000791), and 1300 bp downstream of *DHFR*. Intron-based primers were developed to avoid amplification of pseudo genes and to reveal potential splice site variants. Concerning amplification of both 5′ and 3′ UTRs, primer sets were developed taking into account the homology of the known pseudogenes. In addition, alignment of these primer sets showed no matches with any of the known *DHFR* pseudogenes. PCR amplifications were performed in a total volume of 50 μl on an iCycler (Biorad, The Netherlands). For details, see Table 1. The reaction mixture contained 200 nmol/l of both forward and reverse primer (Biolegio BV, The Netherlands); 200 μmol/l dNTPs, 0.5 U of recombinant *Taq* polymerase (both from Invitrogen, The Netherlands); PCR buffer containing 20 mmol/l Tris-HCl buffer (pH 8.4), 50 mmol/l KCl and 5% DMSO; 1.0–4.0 mmol/l $MgCl_2$ and 75 ng DNA. An alternative PCR buffer, containing 50 mmol/l Tris-HCl pH 9.2, 16 mmol/l $(NH_4)_2SO_4$, 2% DMSO and 0.1 % Tween-20 (Roche Applied Science, Switzerland), was used where necessary (see Table 1). PCR conditions were as follows: initial denaturation of 4 min at 94°C, 35 cycles of 94°C/60 s, 55–65°C/30 s, 72°C/30 s, and a final extension of 7 min at 72°C. The PCR products obtained were analyzed on a 2% agarose gel and subsequently sequenced on an ABI Prism 3730 automated sequencer using the ABI Prism Big Dye Terminator cycle

Table 1 Primer sequences and PCR conditions used for amplification of the *DHFR* gene

Primer	*Sequence (5′ → 3′)*	*Product Size, bp (seq[a])*	T_{ann} (°C)[b]	*[$MgCl_2$] (mM)*
5′ UTR F[b]	CCTCAGCGCTTCACCCAATTTG	450 (−664 to −213)	65	4.0
5′ UTR R	CCGGGCTGCCATCCTTGC			
Exon 1 F[b]	GGAGGAGGAGGTGGATTTC	591 (−358 to 233)	55	2.0
Exon 1 R	GCAGCAGAAAAGGGGAATC			
Exon 2 F	CCCTACCCACAGCGCTCCG	348 (343–691)	60	1.5
Exon 2 R	GCCTGATAATTTGCTCGTGCG			
Exon 3 F	GCATGCAGACTCCACACAGACG	372 (4833–5205)	65	1.5
Exon 3 R	GCAGCTTCATCAATAGCTCC			
Exon 4 F	GTTCTCTCCTGCCCTGTCCAAG	491 (16176–16667)	65	1.5
Exon 4 R	GGCAAGGAAGCTGAAAGTAGAAC			
Exon 5 F	GTAAGCAAACTGGAGGCCAGAC	490 (20245–20735)	65	1.5
Exon 5 R	GCACCCATCATCCTAGCAGTACAC			
Exon 6 F+3′ UTR[b]	GAAACTGCTGACTGGTTTTTGAG	1666 (25036–26702)	65	4.0
Exon 6 R+3′ UTR	GGTTCAAGCAACCATCATCCC			
ISP 3′ UTR1 F	CTAGTTTAAGTTGTTCCCC	− (25417–25803)	58	—
ISP 3′ UTR2 F	CGTGTATATCCAGAGGTTTGTAG	− (25780–26235)	66	—
ISP 3′ UTR3 F	GGGAACAGTGAATGCCAAAC	− (26215–26702)	60	—

DHFR, dihydrofolate reductase; ISP, internal sequence primer; PCR, polymerase chain reaction; T_{ann}, annealing temperature; UTR, untranslated region.
[a]Sequence covered by primer(set), positions are relative to the A in the translation initiation codon.
[b]An alternative buffer (see Materials and methods section) was used for the PCR amplification reaction.

sequence kit according to the instructions of the manufacturer (PE Biosystems, The Netherlands). Newly identified variants were confirmed on both DNA strands by direct sequencing.

Genetic analysis of DHFR 9-bp repeat

We identified a 9-bp repeat in the 5′-upstream region of the *DHFR* gene. We obtained the most frequent alleles (3×, 6× and 7× repeat) by cloning PCR fragments (450 bp) into the pGEM-T vector system and transformation of *Escherichia coli* JM109 High-efficiency Competent cells (Promega, The Netherlands). After ampicillin selection on LB-plates (IPTG/X-gal screening) and plasmid DNA isolation (Wizard plus SV, miniprep kit, Promega), these alleles were used as positive controls in the genotype analyses. In order to facilitate genotyping, a new primer set was chosen for the analysis (F 5′-GCCCAGTCCCAGACAGAAC-3′ and R 5′- GAGCCGATTCTTCCAGTCTAC-3′). The resulting PCR product was 180 bp (for the 6× repeat allele), allowing a clear distinction between the alleles. PCR conditions: 4 min at 94°C, 35 cycles of 94°C/60 s, 59°C/30 s and 72°C/30 s, and a final extension of 7 min at 72°C. The reaction mixture (50 μl) contained 200 nmol/l of both forward and reverse primer (Biolegio BV, The Netherlands); 200 μmol/l dNTPs, 0.5 U of recombinant *Taq* polymerase (both from Invitrogen, The Netherlands); PCR buffer containing 50 mmol/l Tris-HCl (pH 9.2), 16 mmol/l $(NH_4)_2SO_4$, 2% DMSO and 0.1% Tween-20 (Roche Applied Science, Switzerland); 2.0 mmol/l $MgCl_2$ and 75 ng DNA. The PCR products were analyzed on large 3% agarose gels by running the samples at least 4 h at 125 V. In each PCR, DNA samples with a known (sequence confirmed) genotype were run as positive controls. When PCR products were not in control range or difficult to genotype, they were excised and purified from the agarose gel (Gel extraction kit, Qiagen B.V., The Netherlands) and sequenced to identify the length of the allele. Genotype data were obtained from 417 subjects.

Analysis of DHFR intron 1 19-bp deletion

Screening for the 19-bp deletion in intron 1 of the *DHFR* gene was essentially as described previously.[20] In short, the PCR reaction mix (25 μl) contained 75 ng of genomic DNA, 1× Ampli *Taq* gold buffer, 1.5 mmol/l $MgCl_2$, 250 μmol/l dNTPs, 1 μmol/l of each primers (FW1: 5′-CCACGGTCGGG GTACCTGCC-3′, FW2: 5′-ACGGTCGGGGTGGCCGACTC-3′ and RV: 5′-AAAAGGGGAATCCAGTCGG-3′), 1 mol/l betaine and 0.5 U *Taq* polymerase. PCR conditions were as follows: initial denaturation of 4 min at 94°C, 35 cycles of 94°C/60 s, 58°C/60 s, 72°C/60 s, and a final extension of 7 min at 72°C. The PCR products were analyzed on a 3% agarose gel, stained with ethidium bromide and visualized by UV light. On each gel, DNA samples with a known (sequence confirmed) genotype (no del/no del, no del/del, del/del) were run as positive controls. Genotype data were obtained from 330 subjects.

Biochemical parameters

Blood samples were drawn from the antecubital vein for determination of total plasma homocysteine and folate. EDTA samples for homocysteine and folate measurement were placed on ice immediately and centrifuged at 3500 *g* for 5 min with minimal delay. The plasma or serum was separated and stored at −20°C. Total plasma homocysteine concentrations were measured by an automated high-performance liquid chromatography method with reverse

phase separation and fluorescent detection (Gilson 232-401 sample processor, Spectra Physics 8800 solvent delivery system and LC 304 fluorometer).[23] DNA extraction was performed as described previously.[22] Serum and RBC folate concentrations were measured with the Dualcount Solid Phase No Boil Radioassay (Diagnostic Product Corporation, Los Angeles, CA, USA).

Statistics

tHcy and folate concentrations were logarithmically transformed before all analyses, and are expressed as a geometric mean with a 95% confidence interval (CI). Differences in metabolite concentrations were determined by linear regression analysis and expressed as changes relative to the wild type. A two-tailed $P<0.05$ was accepted as statistically significant. For all analyses, SPSS 12.0 for Windows was used.

Results

Human *DHFR* sequence analysis

We sequenced the coding region, including intron–exon boundaries, and UTRs of the *DHFR* gene (nucleotide −664 to 26702 relative to the A in the translation initiation codon) (see Table 1 for details). We observed no genetic variation within the coding region, but did identify a 9-bp

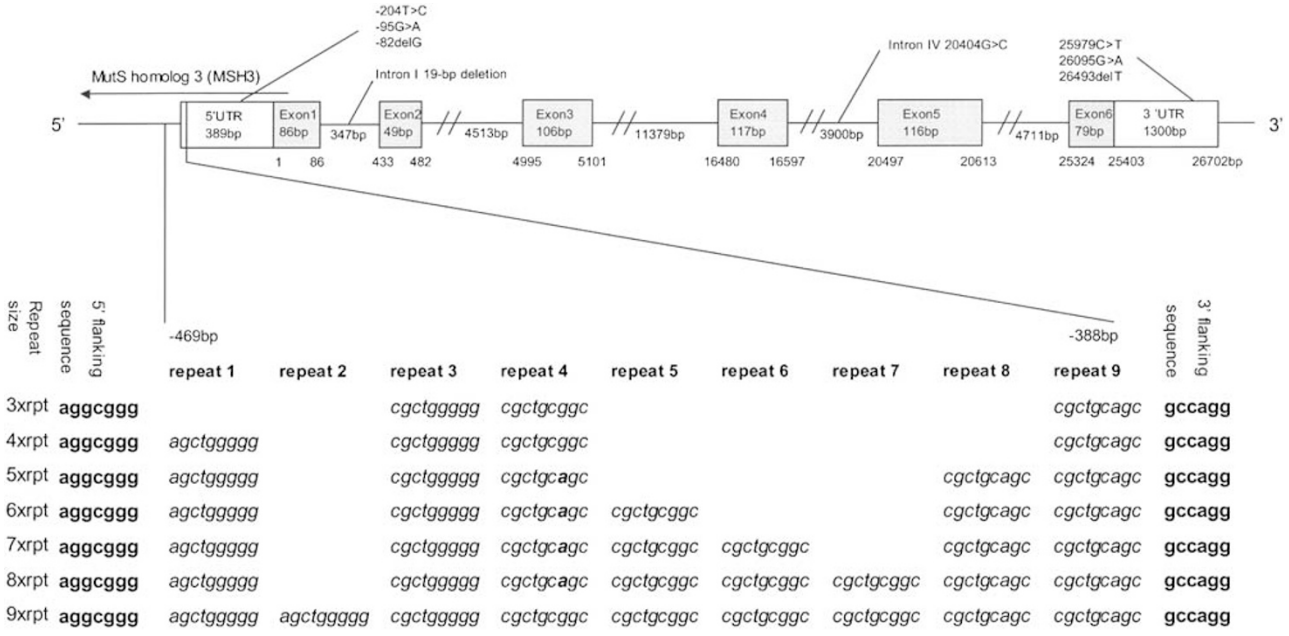

Figure 1 *DHFR* gene structure and DNA sequence of the 5′-upstream region 9-bp repeat as observed for the different alleles.

Table 2 Observed sequence variants in the human *DHFR* gene in 20 subjects

Region	*Location (bp)*	*Variant*	*Allele frequency*
5′ UTR	−388[a]	9-bp repeat[b]	0.29 (3× rpt), 0.62 (6× rpt) 0.09 (7× rpt), <0.01 (4, 5, 8, 9× rpt)
	−204	T>C	0.50 (C)
	−95	G>A	0.13 (A)
	−82	delG	0.13 (delG)
Intron I	146	19-bp deletion[b,c]	0.43 (del)
Intron IV	20 404	G>C	0.03 (C)
3′ UTR	25 508	A>T	0.14 (T)
	25 979	C>T	0.13 (T)
	26 095	G>A	0.13 (A)
	26 493	delT	0.21 (delT)

DHFR, dihydrofolate reductase; rpt, repeat; UTR, untranslated region.
[a]Locations are relative to the A in the translation initiation codon.
[b]Screened in the whole population.
[c]First reported by Johnson *et al*.[20]

repeat in the highly GC-rich 5′-upstream region of *DHFR*. The repeat was located 388 bp upstream of the first nucleotide of the translation initiation codon (−388 bp), and partially overlapped with the 5′ UTR. We observed alleles ranging from 162 bp (ie 3 × repeat) to 216 bp (ie 9 × repeat), of which the determined DNA sequences are shown in Figure 1. The most common genotypes (%) were 3/3 (10.6), 3/6 (30.9), 6/6 (41.0) and 6/7 (9.6). Less frequent genotypes (%) were 3/7 (4.1%), 6/9 (1.4%), 3/5, 3/8, 5/6, 6/8 (all 0.5%), 4/7 and 7/7 (both 0.2%). The general consensus sequence of the repeat was **A/C**GCTG**G/CG/A**G**G/C**. In addition, we found three single-nucleotide polymorphisms in the 5′ UTR, one in intron IV and three in 3′ UTR (see Table 2).

Association between DHFR variants and tHcy and folate

Because the 9-bp repeat is located in the promoter region and the 19-bp deletion is thought to affect gene expression,[20] we only screened these potentially most important variants for their effect on tHcy, serum and RBC folate in our study population. As shown in Table 3, the 9-bp repeat was not associated with tHcy in our study population. Although the 3/6 and 3/7 repeat compared to the 3/3 repeat genotype showed a trend towards higher plasma folate levels (P trend = 0.015 for the 3/3, 3/6 and 3/7 repeat genotype), this increment did not persist in the 6/6 and 6/7 repeat genotypes. The 19-bp deletion was associated with a 2.5% (95% CI: −10.8 to 6.5) and 14.4% (95% CI: −23.5 to −4.5) decrease in tHcy for the heterozygous and homozygous genotype, respectively, compared with the wild-type genotype (P ANOVA = 0.016)(Table 3). Adjustment for age and sex did not change the association between this deletion and tHcy. Serum and RBC folate levels were similar between the genotypes. No interaction between the 9-bp repeat and 19-bp deletion or between low folate (<30th percentile, 10.0 nmol/l) and either variant on tHcy was observed (not shown).

Discussion

In this study, we searched for genetic variation in the *DHFR* gene that may affect tHcy, serum and RBC folate levels. We identified a 9-bp repeat in the 5′-upstream region of *DHFR* that partially overlapped with the 5′ UTR. The 9-bp repeat may affect mRNA stability or translation efficiency, similarly as described for the 28-bp repeat in the functionally related thymidylate synthase.[24] The repeat appeared highly polymorphic with alleles ranging from 3 to 9 repeats. In 1995, Nakajima *et al*.[25] described, in Japanese subjects, a 9-bp repeat in exon 1 of the DNA repair gene mutS homolog3, which partially overlaps with the *DHFR* gene and may share promoter elements. Our repeat most likely represents the same repeat, as the consensus

Table 3 Association between the *DHFR* variants and crude plasma tHcy and folate levels

DHFR variant	*Genotype*	*Controls*, n (%)	*Mean tHcy, μmol/l (95% CI)*	*Relative change tHcy, % (95% CI)*	*Mean folate, nmol/l (95% CI)*	*Relative change folate, % (95% CI)*	*Mean RBC folate, nmol/l (95% CI)*	*Relative change RBC folate, % (95% CI)*
9-bp repeat[a]	3/3	44 (10.6)	10.6 (9.4 to 11.8)	0[b]	11.9 (10.3 to 13.7)	0[b]	371 (325 to 425)	0[b]
	3/6	129 (30.9)	10.4 (9.2 to 11.9)	−1.3 (−13.2 to 12.4)	13.5 (11.5 to 16.1)	14.6 (−3.0 to 35.4)	423 (362 to 494)	13.9 (−2.6 to 33.1)
	3/7	17 (4.1)	10.2 (8.2 to 12.6)	−3.5 (−22.0 to 19.2)	15.1 (11.5 to 19.8)	27.1 (−3.1 to 66.9)	388 (302 to 498)	4.5 (−18.6 to 34.2)
	6/6	171 (41.0)	10.3 (9.1 to 11.7)	−2.2 (−13.8 to 10.8)	13.0 (11.0 to 15.2)	9.2 (−7.0 to 28.3)	405 (348 to 371)	9.0 (−6.3 to 26.7)
	6/7	40 (9.6)	11.0 (9.0 to 12.5)	0.4 (−14.7 to 18.1)	12.8 (10.4 to 15.8)	7.6 (−12.5 to 32.6)	416 (342 to 505)	12.0 (−7.9 to 35.9)
19-bp deletion	no del/no del	114 (34.5)	10.9 (10.2 to 11.6)	0[b]	13.5 (12.5 to 14.8)	0[b]	386 (357 to 418)	0[b]
	no del/del	150 (45.5)	10.6 (9.6 to 11.6)	−2.5 (−10.8 to 6.5)	13.5 (12.0 to 15.1)	−0.6 (−11.5 to 11.5)	398 (358 to 442)	+2.9 (−7.4 to 14.3)
	del/del	66 (20.0)	9.2 (8.0 to 10.4)[c]	−14.4 (−23.4 to −4.5)[c]	12.8 (11.1 to 14.8)	−5.8 (−18.5 to 8.8)	388 (340 to 442)	+0.4 (−12.0 to 14.3)

CI, confidence interval; DHFR, dihydrofolate reductase; RBC, red blood cell; tHcy, total homocysteine.
[a]Only the genotypes with a frequency of >2.5% are shown.
[b]Reference category.
[c]P ANOVA <0.05.

sequence highly resembled that reported by Nakajima, although higher repeat sizes were present in our, predominantly Caucasian, population. The repeat may have arisen from duplications of one (or more) ancestral allele(s) or result from DNA replication errors. Despite its location in the promoter region, the length of this repeat was not associated with tHcy, serum and RBC folate levels.

In 2001, Goto *et al*.[26] reported a functional variant (829C>T) in the 3′ UTR of *DHFR*. We did not observe this variant in the subjects that were included in the sequence analysis, suggesting that it is a population-specific polymorphism or has a minor allele frequency below 2.5%. Johnson *et al*.[20,27] identified a 19-bp deletion in the highly conserved intron 1, which may be a maternal risk factor for having a child with spina bifida and preterm delivery. It has been postulated that this variant deletes a putative SP1 transcription factor-binding site (required for transcriptional activation/repression) or may affect the splicing process. We show that the 19-bp del/del genotype is associated with a reduced plasma tHcy of about 1.5 μmol/l (14%) compared to wild-type subjects. This may suggest that the 19-bp deletion indeed increases *DHFR* expression thereby facilitating homocysteine remethylation. Unfortunately, no RNA was available from our study population to support its effect at the expression level, which warrants further investigation.

We detected several other variants in non-coding regions of DHFR which, based on the sequence, could not be ascribed to known pseudogenes. The main part of the introns has not been screened, but these may contain functional variants as well. It is possible that intronic polymorphisms represent a functional variant, although the intronic sequences, except for intron 1, are not well-conserved across species.[28] We assessed linkage disequilibrium (LD) between the 19-bp deletion and the most common alleles of the 9-bp repeat (3× and 6×), and found them to be in almost complete LD (D′ 0.9). However, the (squared) correlation coefficient was low ($R^2 = 0.3$). The HapMap consortium investigated LD between existing DHFR single-nucleotide olymorphisms (http://www.hapmap.org/), and showed that one haplotype block of about 24 kb almost completely covers the *DHFR* gene. This suggest that the number of variants of the *DHFR* gene present in the population is limited and may be captured by genotyping just a small number of polymorphisms in the gene.

In conclusion, the coding region of *DHFR* harbors no variation in our population, which illustrates its importance in folate metabolism. Several variants were found within the non-coding regions of *DHFR*, potentially the most interesting being the 9-bp repeat in 5′ UTR. The 19-bp deletion in intron 1 was associated with lower tHcy levels and may be a genetic risk factor for cardiovascular disease and cancer, in addition to its reported effect on neural tube defect risk.

Acknowledgements

This study was supported by Grant 2002B68 from The Netherlands Heart Foundation. Martin den Heijer, MD, PhD, is supported by a VENI-grant from the Dutch Organization for Scientific Research (NWO).

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