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Identification of four novel mutations in severe methylenetetrahydrofolate reductase deficiency

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Severe methylenetetrahydrofolate reductase (MTHFR) deficiency is an inborn error of folate metabolism, and is inherited as an autosomal recessive trait. MTHFR is a key enzyme in folate-dependent remethylation of homocysteine, and reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Patients with this severe enzymatic deficiency are biochemically characterised by homocystinuria and hypomethioninaemia, and may suffer from neurological abnormalities, mental retardation and premature vascular disease. Here we report the molecular basis of severe MTHFR deficiency in four unrelated families from Turkish/Greek ancestry. By use of reverse-transcriptase (RT)-PCR, subsequently followed by direct sequencing analysis, we were able to identify four novel mutations in the *MTHFR* gene: two missense (983A→G; 1027T→G) and two nonsense (1084C→T; 1711C→T) mutations. Furthermore, a splice variant containing a premature termination codon, was observed in one patient, probably as a secondary effect of the 1027T→G missense mutation. The ongoing identification and characterisation of mutations in the *MTHFR* gene will provide further insight into the heterogeneity of the clinical phenotype in severe MTHFR deficiency.

Keywords: methylenetetrahydrofolate reductase; mutations; inborn error of metabolism; folate; splice variant

Introduction

Methylenetetrahydrofolate reductase (MTHFR) [EC 1.5.1.20] is a key enzyme in the folate-dependent remethylation of homocysteine. The enzyme reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulating form of folate, and donor of the methyl group in the remethylation of homocysteine to methionine. In addition to its involvement in protein synthesis, methionine is converted to S-adenosylmethionine (AdoMet), the most predominant methyl donor in the human body. MTHFR, a flavoprotein, is allosterically regulated by AdoMet; high levels of AdoMet inhibit MTHFR activity and thereby the remethylation of homocysteine to methionine.[1,2]

MTHFR deficiency, the most common inborn error of folate metabolism,[3,4] is inherited as an autosomal recessive trait and may be subdivided into severe and thermolabile MTHFR deficiency. Patients with the

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severe form have severely elevated homocysteine levels in blood and urine, a decreased concentration of methionine in plasma, and display a wide range of clinical features, such as neurological abnormalities, mental retardation, and premature vascular disease. Residual MTHFR activity in cultured fibroblasts is usually < 20% compared with controls, whereas virtually no residual activity can be detected in isolated lymphocytes. The variety in clinical presentation, age of onset, and residual MTHFR activity in cultured fibroblasts, suggest the existence of a strong heterogeneity in mutations causing severe MTHFR deficiency.

The recent localisation of the *MTHFR* gene on chromosome 1 and the elucidation of the MTHFR cDNA[5] permitted the molecular characterisation of patients with severe MTHFR deficiency. Goyette *et al* reported the identification of mutations in the MTHFR gene in patients with a homozygous MTHFR deficiency,[5–7] and correlated genotypes to residual MTHFR activity in cultured fibroblasts and to clinical phenotype.[6]

Recently, Kang *et al* described a thermolabile variant of MTHFR with a specific MTHFR activity in isolated lymphocytes < 50% of the control mean and with increased thermolability after heat incubation for 5 min at 46°C.[8,9] This thermolabile variant may result in mildly elevated homocysteine concentrations and is reported to be associated with cardiovascular disease.[10,11] In 1995 we described a 677C→T transition responsible for the thermolabile MTHFR variant.[12] Recent studies have investigated this mutation as a genetic risk factor for spina bifida[13–15] and cardiovascular disease.[16–20] All studies indicate that homozygotes for this transition have mildly elevated plasma homocysteine, especially in circumstances of low folate status[21,22]

In this report, we describe the identification of two missense and two nonsense mutations in the *MTHFR* gene in four unrelated families from Turkish/Greek ancestry with severe MTHFR deficiency. Furthermore, our data contribute to the genotype/phenotype correlations in this metabolic disease.

Patients, Materials and Methods

Patients

Patient CM was the second girl of a family of Greek-Macedonian ancestry, and was admitted to our hospital at the age of 2 because of marked psychomotor retardation. This patient has been described before as a case report by Hyland *et al*[23] (case 2) and by Wendel and Bremer.[24] Both parents originate from the same village, but without known consanguinity. The girl was microcephalic, hypotonic, restless, and was unable to sit without support. Plasma homocystine concentrations were in the range of 15–25 μm/L, homocysteine-cysteine mixed disulphide 25–35 μm/L, and methionine concentrations 4–5 μm/L. Diagnosis of severe MTHFR deficiency was established by absence of MTHFR activity in extracts of cultured fibroblasts. Therapy was initiated, and she received 20 g of betaine and 15 mg of folinic acid daily. Plasma methionine levels increased to > 25 μm/L, and plasma homocystine concentration decreased to trace amounts. Her clinical condition improved substantially; however, now at 17 years of age, she is still severely mentally retarded.

Patient UB, a male, is the seventh child of a healthy consanguineous Turkish couple. In at least three previous children of the family, MTHFR deficiency had been diagnosed. In this child, MTHFR was diagnosed immediately after birth with plasma homocystine concentrations of 15–27 μm/L, and methionine concentrations of 4–6 μm/L. Therapy with betaine (60 mg/kg body weight, daily) was initiated at the sixth day of life. Plasma methionine levels increased to > 25 μm/L, and plasma homocysteine decreased to trace amounts. He was again referred to our hospital at the age of 4 years, when he was found to be severely mentally retarded, and showed signs of dysmyelination in a cranial MRI. During the four years, betaine has only been given irregularly, and even dropped for long periods of time. When regular treatment with betaine was restarted, total plasma homocystine decreased to 80–100 μm/L, and methionine increased to high normal values.

Patient K was a male child of a healthy consanguineous Turkish couple, previously presented as case 3 in Hyland *et al*.[23] Homocystinuria with low plasma methionine was diagnosed at the end of the first month of life; however, the patient was not treated. A CAT scan revealed severe internal hydrocephalus, being rapidly progressive during the first months. At 10 months of age, he suffered from severe psychomotoric retardation, severe muscular hypotonia, and he had no social contact. Plasma homocystine (38–50 μm/L) and homocysteine–cysteine mixed disulphide (40–60 μm/L) concentrations were severely elevated, whereas plasma methionine concentrations were decreased and ranged from 6–10 μm/L. Specific MTHFR activity was assayed in extracts of cultured fibroblasts, but was undetectably low. Betaine (15–20 g daily) treatment was initiated. As

a result, plasma methionine concentrations increased to > 30 μm/L, and homocystine levels decreased to < 5 μm/L; homocysteine–cysteine mixed disulphide remained virtually unchanged. On treatment, the patient showed some neurological improvement. He became more alert and gained some social contact, but was never able to walk. At the age of 7 he died severely mentally retarded with hyperpyrrhexia.

Patient U is an infant first child born from a consanguineous Turkish couple. At the age of 10 months, she presented with severely retarded psychomotor development, severe microcephaly, and homocystinuria.[25] Methionine concentration in plasma was < 5 μm/L and total plasma homocysteine was > 150 μm/L. Upon betaine treatment (6–9 g daily), her plasma methionine increased to normal values, and total plasma homocysteine decreased to 90 μm/L. Clinically, her condition improved remarkably. However, now at the age of 4, she is still severely mentally retarded.

MTHFR Assay

MTHFR activity was determined radiochemically in its reverse direction.[26] In the assay ^{14}C Me-THF served as a cosubstrate in the presence of menadione as electron acceptor. Fibroblasts were resuspended in 50 μM potassium phosphate buffer (pH 7.2), sonicated on ice, and subsequently centrifuged for 40 min at 15 800 g at 4°C. The incubation mixture consisted of 0.18 M potassium phosphate buffer (pH 6.8), 1.15 μM EDTA (pH 7.0), 11.5 μM ascorbic acid, 54 μM FAD, 17 μM ^{14}C Me-THF ($\sim 3.0 \times 10^5$ dpm), 3.5 μM menadione, and a maximum of 250 μl enzyme extract in a total volume of 600 μl. Menadione was added to start the incubation. The incubation was performed for 20 min at 37°C in the dark, and was stopped by addition of 10 μl of 1.0 M formaldehyde, 50 μm dimedone in 200 μl ethanol:water (1:1), and 100 μl 3.0 M potassium acetate (pH 4.5). The incubation mixture was heated for 15 minutes at 95°C and quenched on ice for at least 10 min. After addition of 3 μl toluene, the mixture was shaken vigorously for 15 s and centrifuged at low speed. A 2.0 μl aliquot of the toluene phase was used for the measurement of radioactivity. Fibroblasts were cultured as described previously.[27,28] Protein content of the sample was determined by the method of Lowry *et al*.[29] Specific MTHFR activity was expressed in *n*mol formaldehyde/mg protein per hour.

Homocysteine Determination

Determination of methionine and the non-protein bound forms of homocysteine, ie homocystine and homocysteine–cysteine mixed disulphide, in serum of our patients was performed as described earlier.[30] Total plasma homocysteine concentrations (ie total amount of protein and non-protein bound homocysteine) were determined according to Te Poele-Pothoff *et al*.[31]

Mutation Detection

Primers used for PCR amplification of cDNA and genomic DNA sequences are listed in Table 1. Primer sequences are based on the cDNA sequence published by Goyette *et al*,[5] and on the sequence submitted to GenBank (accession number U09806).

Total RNA was extracted from cultured fibroblasts or isolated lymphocytes using the method of Chomczynski and Sacchi.[32] Five μg total RNA was reversed transcribed to cDNA as described previously.[33] A 5 μl aliquot of the cDNA product was subjected to PCR amplification in a total volume of 100 μl, consisting of 100 ng forward and reverse primer, 200 μM each dNTP, 10 μM Tris-HCl pH 8.3, 50 μM KCl, 1.5–3 mM $MgCl_2$ and 1 unit Taq polymerase (Life Technologies, Breda, The Netherlands). PCR conditions were as follows: 3 min

Table 1 Oligonucleotides used for PCR amplification and direct sequencing

Oligonucleotide	*Forward/Reverse*	*Sequence (5′ → 3′)*	*Position*[a]
1	F	5′-GCCATGGTGAACGAAGCCAG-3′	10–29
6	R	5′-TGGTAGCCCTGGATGGGAAAG-3′	800–780
7	F	5′-ATCACTTGCCCCATCGTCCC-3′	754–773
10	R	5′-CTCCTTGGGGACTTGCTCTTCAG-3′	1256–1234
14	F	5′-CTTTGGGGAGCTGAAGGACTACTAC-3′	1200–1224
17	R	5′-ATGGAGCCTCCGTTTCTCTCGC-3′	1981–1960
9	F	5′-CCAGGCCTCCACTTCTACACCC-3′	958–979
19	R	5′-CACTTTGTGACCATTCCGGTTTG-3′	1362–1340
15	F	5′-CAGCGGGGGCTATGTCTTCCAG-3′	1518–1539
21	R	5′-CTCCTCTGGGGATCTCTGGGC-3′	intronic

[a]Position according Goyette *et al*[5] and the MTHFR sequence submitted to GenBank (accession number U09806).

Table 2 Genotype and MTHFR activity in cultured fibroblasts of patients with severe MTHFR deficiency

Patient	*Mutations*	*Amino acid change*	*MTHFR activity*[a]	
			+FAD[b]	–FAD
CM	A983G/A983G C677T/C677T	N→S/N→S A→V/A→V	0	0
HB	T1027G/T1027G C677T/C677T splice variant	W→G/W→G A→V/A→V Premature stop codon	0.7	0.2
K	C1084T/C1084T C677T/C677T	R→X/R→X A→V/A→V	0	0
U	C1711T/C1711T	R→X/R→X	2.4	1.7
Healthy controls ($n=6$)			4.9–10.0	3.9–8.6

[a]MTHFR activity is expressed in *n*mol formaldehyde formed per mg protein/h.
[b]FAD is added to the enzyme mixture in a concentration of 54 μM.

initial denaturation at 94°C, followed by 35 cycles of 60 s at 92°C, 60 s at 52–60°C, and 30–120 s at 72°C. A final extension of 7 min at 72°C was performed to ensure a complete extension of all PCR products. The PCR products generated were purified and subjected to direct sequencing analysis using an ABI 377 automated DNA sequencer according to the manufacturer's recommendations (Applied Biosystems Inc., Forster City, CA, USA).

Genomic DNA was extracted from cultured fibroblasts or peripheral blood lymphocytes.[34] Mutations were confirmed on genomic DNA by restriction enzyme analysis of a genomic PCR fragment. Depending on the length of the fragments expected, restriction fragments were separated in either 3% agarose or 10–20% polyacrylamide gels containing 5% glycerol, and visualised after ethidiumbromide staining and UV illumination.

Results

Missense Mutations 983A→G

In patient CM, we identified a homozygous A→G transition at position 983 of the MTHFR cDNA. This mutation results in a putative amino acid change from a conserved asparagine to serine (N→S) in the mature protein, and introduces an additional *Dde*I restriction site Table 2. The mutation was confirmed at genomic DNA level using oligonucleotides 9 and 21 (intronic primer). After *Dde*I restriction enzyme analysis, the mutated allele yielded 3 fragments (54 bp, 42 bp, and 23 bp) whereas the wild-type allele yielded two fragments (77 bp and 42 bp). The parents and an unaffected brother were also tested for this mutation by *Dde*I restriction enzyme analysis. Both parents and the brother were heterozygous for this transition (Figure 1a). The asparagine residue involved is a conserved amino acid in several oxidoreductases in both prokaryotic and eukaryotic organisms (Figure 1b). The index patient was also found to be homozygous for the previously described 677C→T transition, related to thermolability of the protein. The remainder of the cDNA, which was sequenced entirely, was identical to the wild-type sequence, except for some silent mutations (data not shown). The 983A→G mutation was not observed in seven other patients with severe MTHFR

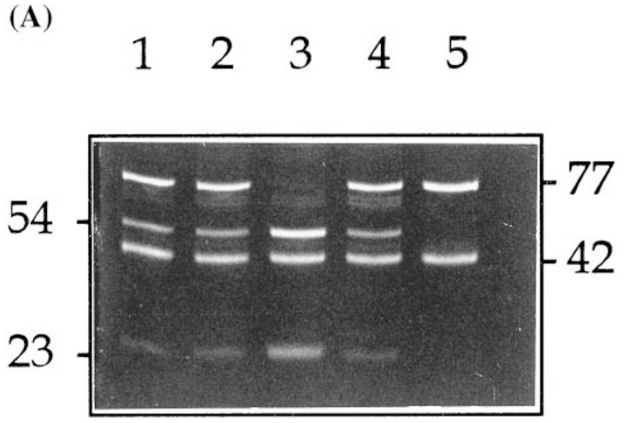

Figure 1a *Screening of 983A→G (N→S) mutation in the pedigree of patient CM. A genomic DNA fragment was amplified by PCR, screened for the mutation by* Dde*I restriction enzyme analysis, and subsequently electrophoresed in a 20% polyacrylamide gel. The mutated allele yields three fragments (54 bp, 42 bp, and 23 bp), whereas the wild type allele displays two fragments (77 bp and 42 bp). Lane 1: father; lane 2: mother; lane 3: index patient; lane 4: brother; lane 5: healthy control.*

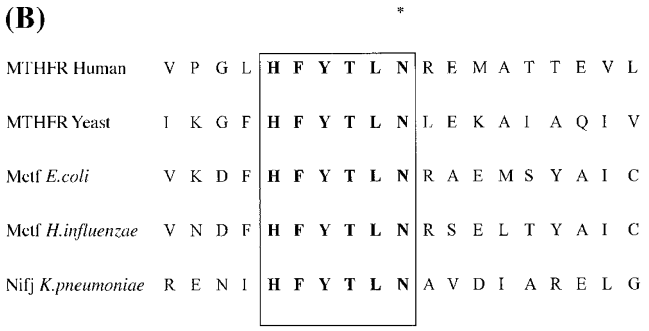

Figure 1b *Alignment of different MTHFR proteins and one oxidoreductase from eukaryotic and prokaryotic species. Absolute conservation is indicated in bold and is boxed. Amino acids are indicated in one letter code, and the asparagine residue (N) mutated in our index patient is indicated with an asterisk. Metf: methylenetetrahydrofolate reductase; Nifj: pyruvate-flavodoxin oxidoreductase.*

deficiency and was absent in 40 healthy unrelated Dutch controls.

Missense Mutation 1027T→G

In patient UB a homozygous 1027T→G transversion was observed, converting a non-conserved tryptophan (W) residue into a glycine (G). The mutation creates a *Bsl*I restriction site which was used to confirm the mutation at genomic DNA (data not shown). By amplifying cDNA in the region containing this mutation, we detected a splice variant of a comparable abundance compared with the normal cDNA fragment. This splice variant was approximately 230 bp longer than expected from the sequence published by Goyette *et al*.[5] Sequencing analysis of both cDNA fragments and the genomic equivalent revealed the retainment of an intronic sequence at position 1043 in the longer fragment. The intron contained a preliminary translation termination codon (Figure 2). Direct sequencing of both donor and acceptor splice sites and the entire intron in our patient and a control did not reveal any sequence aberrations as a possible explanation of alternative splicing. This mutation was not detected among seven other MTHFR deficient patients or 40 Dutch controls. The patient is a homozygote for the 677C→T mutation.

Nonsense Mutation 1084C→T

In patient K, we observed a homozygous C→T transition at position 1084 of the cDNA, resulting in a CGA (R) to TGA (X) codon change. This mutation introduces a premature stop codon, probably resulting in a truncated protein. The mutation creates an additional *Dde*I restriction site, which facilitates screening of this

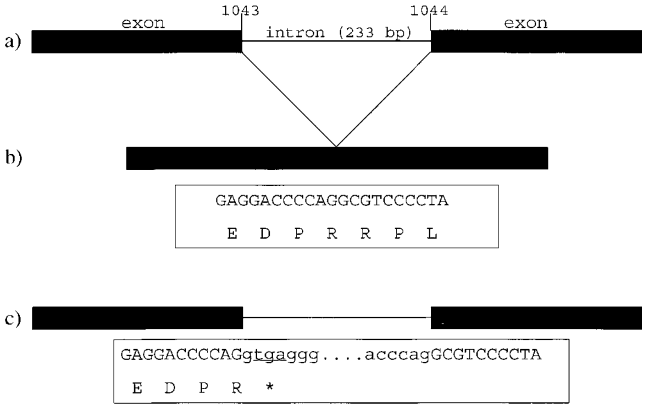

Figure 2 *Overview of splice junction sites at cDNA positions 1043 (splice donor site) and 1044 (splice acceptor site).* **a** *Schematic illustration of genomic DNA fragment.* **b** *Normal splice variant. DNA and amino acid sequences are boxes.* **c** *Aberrant splice variant, in which intron sequence is retained in transcript. Exonic DNA sequences are indicated in upper case, intronic in lower case. The premature translation termination codon (tga) is underlined and indicated with an asterisk in the amino acid sequence. In patient UB we observed both normal and aberrant splice variant in equal abundance.*

transition on genomic DNA level. Analysis of this mutation on a genomic PCR fragment confirmed the presence of the mutation in the patient, also demonstrating the homozygous state for this transition. Family members were not available for DNA analysis, so compound heterozygosity for a deleted allele and the nonsense mutation could formally not be excluded. Seven other patients with an enzymatically homozygous MTHFR deficiency were tested by *Dde*I restriction enzyme analysis; all were negative for this mutation. Furthermore, the patient was homozygous for the 677C→T transition.

Nonsense Mutation 1711C→T

In patient U, we detected a 1711C→T nonsense mutation in homozygous state, which resulted in a codon change from CGA (R) to TGA (X). The premature stop codon will, during translation, most likely result in a truncated protein, in which the 89 carboxy-terminal amino acid residues are deleted. The mutation created an additional *Hph*I restriction site, which was used to confirm the mutation at genomic DNA level in this patient and his parents. Both parents were heterozygous for this transition (data not shown). This mutation was not detected among seven other MTHFR deficient patients. This patient is not a carrier of the 677C→T mutation.

Discussion

In this study, we have investigated four unrelated patients with severe hyperhomocysteinaemia due to MTHFR deficiency, and found two missense and two nonsense mutations in the MTHFR cDNA, which are unique for the specific family.

We observed a 983A→G transition, which resulted in an amino acid substitution from asparagine to serine in the mature protein, both uncharged polar amino acids. The asparagine residue is located in a highly conserved region in MTHFR proteins of both eukaryotic and prokaryotic origin (Figure 1b), an indication of importance in biological structure or function of the protein. Goyette *et al*[6] detected a mutation in the same region affecting the adjacent arginine residue (985C→T; R→C). Their patient showed a relatively mild clinical phenotype, with a high residual enzyme activity (20%) in cultured fibroblasts. Based on the absence of flavin adenine dinucleotide (FAD) stabilisation of the MTHFR protein in their patient during heat inactivation,[26] it was postulated that this region of the protein is critical in FAD binding. Our patient showed a more severe clinical phenotype with no residual enzyme activity in cultured fibroblasts, regardless of FAD addition. Obviously, the aspargine residue seems, in addition to its putative involvement in FAD binding, to be more critical in its catalytic function than the adjacent arginine.

In patient UB we observed a homozygous 1027T→G transversion (W→G) and two splice variants: one fragment of normal size, and one in which the intron at cDNA position 1043 is retained. Both splice variants were of approximately equal abundance. Direct sequencing of the donor and acceptor splice sites in our patient and in a control did not reveal any sequence aberrations which could explain this phenomenon. Both sites are highly homologous to the consensus splice site sequences.[35] Alternative splice site selection as a secondary effect of mutations has been described in the fibrillin (*FBN1*) gene,[36] collagen IVα5 chain (*COL4A5*) gene[37] and in the transacylase (*E2*) gene of the human branched-chain α-keto acid dehydrogenase (BCKAD),[38] in all cases due to a nonsense mutation or frameshift mutation introducing a premature translation termination codon. Theoretically, we could have missed a mutation in one of the up- or downstream located introns as the primary cause of this alternative splicing. Alternatively, the 1027T→G transversion, 16 basepairs upstream of the splice donor site, might have an effect on the global secondary structure of the primary transcript, which could result in a less efficient and variable splicing on both alleles.

The missense mutations described above were the only amino acid changing DNA aberrations observed in our patients. Because control individuals of the same ethnic background were not available for testing, we analysed 40 unrelated Dutch controls for the 983A→G and 1027T→G mutations. Their absence among those individuals, suggests that they are most likely not benign polymorphisms, although only *in vitro* expression of those mutations will be conclusive in demonstrating their pathogenicity.

We observed two nonsense mutations, 1084C→T and 1711C→T, in homozygous form in two unrelated patients, both affecting an arginine residue in the protein; both could result from a deamination of a methylated cytosine to thymidine in a CpG dinucleotide, a common genetic cause of C to T transitions.[39] Because of the introduction of a termination codon, these transitions are expected to be pathogenic. The first mutation, 1084C→T, is located in the putative KRREED linker sequence between the catalytic NH_2-terminal domain and the regulatory COOH-terminal domain.[5] In porcine, this hydrophilic, and highly charged linker region, is susceptible to trypsinisation, resulting in a MTHFR protein which is catalytically active but cannot be regulated by S-adenosylmethionine (AdoMet).[40] Our nonsense mutation is expected to result in a truncated protein, in which only the catalytic domain is translated. Analogous to the porcine MTHFR, this could result in a catalytically active protein. However, residual enzyme activity in cultured fibroblasts of our patient was undetectably low, suggesting that this mutation prevents formation of a catalytically active enzyme or the truncated protein is very rapidly degraded by proteolytic enzymes.[41]

The second nonsense mutation 1711C→T, observed in patient U in homozygous state, results in a truncated protein, in which 89 carboxy-terminal amino acid residues are deleted. None of the prokaryotic *Met*F genes shows any homology with the C-terminal region of the human MTHFR,[5] indicating that this region is probably not involved in catalytic activity or binding of co-factors and substrates. The C-terminal part of the MTHFR protein is only conserved in yeast MTHFR and is probably involved in AdoMet binding. The mutation leads to a biochemical phenotype with relatively high residual enzyme activity in cultured fibroblasts. Obviously, the 89 amino acids do not contain

critical residues for catalytic activity. AdoMet inhibition of the MTHFR activity was not assessed in this patient. However, a defective binding and inhibition of AdoMet due to this mutation would not cause hyperhomocysteinaemia and hypomethioninaemia but would result in a hypermethioninaemia and low to normal homocysteine concentrations.

All patients were treated with high concentrations of betaine (trimethylglycine) to stimulate betaine-dependent remethylation of homocysteine. Although there was a considerable reduction in homocysteine concentration in all patients, total plasma homocysteine levels remain at concentrations (approx. 80 μmol/L) considered to confer a tremendously high risk on arteriosclerosis and thrombosis. In the human body, betaine is converted to dimethylglycine by betaine:homocysteine methyltransferase (BHMT), an enzyme which is only catalytically active in the human liver and possibly kidney.[42] Dimethylglycine can further be demethylated by dimethylglycine dehydrogenase to sarcosine, and via sarcosine dehydrogenase to glycine; the latter can even be catabolised to ammonia and a one-carbon moiety, carbon dioxide, via the glycine cleavage system.[43] In these subsequent reactions one-carbon units are used for the conversion of tetrahydrofolate to 5,10 methylenetetrahydrofolate. The use of high doses of betaine may cause accumulation of 5,10 methylenetetrahydrofolate and depletion of tetrahydrofolate. Because dimethylglycine is an inhibitor of BHMT *in vivo*,[44] administration of folic acid may be recommended in combination with betaine in patients with severe MTHFR deficiency to enhance further conversion of dimethylglycine. Furthermore, high concentrations of 5,10 methylenetetrahydrofolate may stimulate residual MTHFR activity and increase homocysteine remethylation to methionine.

In conclusion, by RT-PCR and direct sequencing analysis, we have identified four novel mutations in the *MTHFR* gene in patients with severe MTHFR deficiency; two missense (983A→G, 1027T→G) and two nonsense mutations (1084C→T, 1711C→T). In one patient we also detected a splice variant, containing a preliminary translation termination codon. All point mutations were present in the homozygous state and were restricted to one pedigree. Although the clinical manifestation of MTHFR deficiency in our patients is diverse, all were diagnosed within the first two years of life, and adequate treatment was instituted. The latter may preclude conclusions regarding clear genotype–phenotype correlations in our patients. Expression of these mutations in either prokaryotic or eukaryotic expression systems should reveal the exact pathogenic mechanism of these mutations in protein structure or enzyme activity.

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