

Glycine *N*-methyltransferase deficiency: A new patient with a novel mutation

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Summary: We report studies of a Greek boy of gypsy origin that show that he has severe deficiency of glycine *N*-methyltransferase (GNMT) activity due to apparent homozygosity for a novel mutation in the gene encoding this enzyme that changes asparagine-140 to serine. At age 2 years he was found to have mildly elevated serum liver transaminases that have persisted to his present age of 5 years. At age 4 years, hypermethioninaemia was discovered. Plasma methionine concentrations have ranged from 508 to 1049 $\mu\text{mol/L}$. Several known causes of hypermethioninaemia were ruled out by studies of plasma metabolites: tyrosinaemia type I by a normal plasma tyrosine and urine succinylacetone; cystathionine β -synthase deficiency by total homocysteine of 9.4–12.1 $\mu\text{mol/L}$; methionine adenosyltransferase I/III deficiency by *S*-adenosylmethionine (AdoMet) levels elevated to 1643–2222 nmol/L; and *S*-adenosylhomocysteine (AdoHcy) hydrolase deficiency by normal AdoHcy levels. A normal plasma *N*-methylglycine concentration in spite of elevated AdoMet strongly suggested GNMT deficiency. Molecular genetic studies identified a missense mutation in the coding region of the boy's GNMT gene, which, upon expression, retained only barely detectable catalytic activity. The mild hepatitis-like manifestations in this boy are similar to those in the only two previously reported children with GNMT deficiency, strengthening the likelihood of a causative association. Although his deficiency of GNMT activity may well be more extreme, his metabolic abnormalities are not strikingly greater. Also discussed is the metabolic role of GNMT; several additional metabolite abnormalities found in these patients; and remaining questions about human GNMT deficiency, such as the long-term prognosis, whether other individuals with this defect are currently going undetected, and means to search for such persons.

Glycine *N*-methyltransferase (GNMT) catalyses the transfer of the methyl group of *S*-adenosylmethionine (AdoMet) to glycine, forming *N*-methylglycine (sarcosine), an activity first described by Blumenstein and Williams in 1960. Sarcosine is normally rapidly metabolized by sarcosine dehydrogenase to methylenetetrahydrofolate and glycine. The result is a cycle in which glycine and cytosolic methylenetetrahydrofolate (via methyltetrahydrofolate, methionine, and AdoMet) are converted back to glycine and mitochondrial methylenetetrahydrofolate. However, several lines of evidence indicate that GNMT has an important role in normal metabolism: Kerr (1972) showed that GNMT activity accounted for most of the removal of AdoMet added to crude extracts of liver, kidney and pancreas of adult rabbits or rats. GNMT has been reported to be present in relatively high amounts: 0.5–3% of soluble protein in adult liver (Cook and Wagner 1981; Kerr and Heady 1974). Studies of sarcosine excretion by humans genetically deficient in their capacity to catabolize sarcosine indicate that formation of this compound increases as the dietary intake of labile methyl groups (as methionine and/or choline) exceeds the normal needs for ongoing AdoMet-dependent methylation reactions, and that the increase in sarcosine formation is sufficient to account for the “excess” methyl intake (Mudd et al 1980). These lines of evidence have been interpreted to suggest that GNMT activity serves to regulate the relative concentrations of AdoMet and *S*-adenosylhomocysteine (AdoHcy) (Kerr 1972), the ratio of which affects the rates of many methyltransferase reactions (Clarke and Banfield 2001); or, alternatively, to permit the non-wasteful removal of excess methionine or AdoMet by methylation of glycine, with subsequent regain of the glycine itself and of the one-carbon group as methylenetetrahydrofolate. Furthermore, several of its kinetic features make GNMT especially suited for removal of excess AdoMet: GNMT activity is hyperbolic with respect to AdoMet concentration, is relatively insensitive to inhibition by AdoHcy (Clarke and Banfield 2001; Kerr and Heady 1973; Ogawa and Fujioka 1982; Yeo et al 1999), and is specifically inhibited by 5-methyltetrahydrofolate pentaglutamate (Wagner et al 1985; Yeo et al 1999). As a result, when formation of the latter 5-methyl derivative is curtailed by the effect of AdoMet on methylenetetrahydrofolate reductase (Jencks and Matthews 1991; Kutzback and Stokstad 1967; Matthews and Daubner 1982), inhibition of GNMT tends to be relieved, and enhanced methylation of glycine promotes AdoMet removal.

Recently, the first proven cases of genetically determined GNMT deficiency in humans were described in two Italian siblings (Luka et al 2002; Mudd et al 2001a). These children had persistent elevations of plasma methionine without elevated plasma total homocysteine (tHcy) (ruling out cystathionine β -synthase (CBS) deficiency) or tyrosine (ruling out tyrosinaemia). Plasma AdoMet was markedly elevated, ruling out methionine adenosyltransferase I/III (MAT I/III) deficiency. They had hepatocellular disease not severe enough to explain their hypermethioninaemia and not attributable to other known causes of hepatitis. Importantly, they did not have elevations of plasma sarcosine, thus raising the possibility of GNMT deficiency—individuals with elevated AdoMet due, for example to methionine ingestion or CBS deficiency, and presumed to have normal GNMT activities,

do have elevated plasma sarcosine. The possible GNMT deficiency was then confirmed by demonstration that each sibling is a compound heterozygote for two inactivating mutations in their GNMT genes.

We report here clinical, metabolic and genetic studies of a third child with GNMT deficiency, in this case due to apparent homozygosity for a novel inactivating mutation in *GNMT*.

MATERIALS AND METHODS

Metabolite assays: Amino acids were assayed by high-performance liquid chromatography using ion-exchange chromatography with a postcolumn derivatization system. Plasma total homocysteine (tHcy) was measured by a fluorescent polarization method (IMX, Abbott Laboratories, Abbott Park, Illinois). Urinary organic acids were assayed by gas chromatography–mass spectrometry. Plasma methionine and tHcy were assayed also by capillary gas chromatography, as were cystathionine, total cysteine (tCys), dimethylglycine and sarcosine (*N*-methylglycine) (Allen et al 1993; Stabler et al 1987, 1993). Plasma AdoMet and AdoHcy (Capdevila and Wagner 1998), and phosphatidylcholine, free choline and betaine (Koc et al 2002) were assayed as described.

Patient and control genomic DNA: Genomic DNA from the patient was isolated from blood samples using NucleoSpin Blood Mini Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. The DNA sample was adjusted to contain 10–20 ng/μl, as determined by absorbance at 260 nm. Ten samples of DNA from apparently healthy American individuals were used as controls. All general manipulations with DNA and PCR products were done according to standard protocols for DNA cloning and analysis (Sambrook et al 1989).

Analyses of the GNMT genes of the patient and controls: A 1.9 kb 5'-fragment of the GNMT gene was prepared by PCR using primers H53 and H34 (Table 1) and a 1.8 kb 3'-fragment was prepared using primers H52 and H36. PCR was done on the RoboCycler 40 (Stratagene, La Jolla, CA, USA) as follows: hot start 94°C for 1 min, followed by 40 cycles at 94°C for 1 min, at 50°C for 1 min, at 68°C for 2 min, and a final extension step at 68°C for 8 min. All PCR reactions were done with PCR superMix High Fidelity reaction mixture (Gibco BRL, Maryland, USA). Concentrations of the primers and genomic DNA were 200 nmol/L and 4 ng/μl, respectively. PCR fragments were purified by QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) or by preparative electrophoresis and elution of the desired fragment from the gel by QIAquick Gel Extraction Kit (QIAGEN), according to the protocol in the kit.

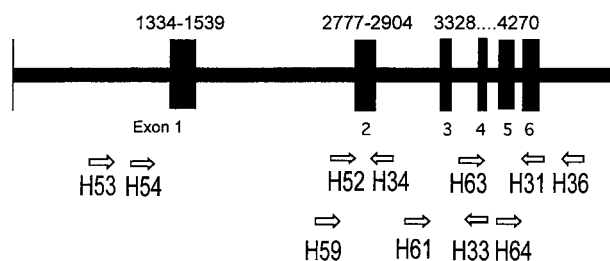
Sequencing of DNA: DNA sequencing was done by the dideoxy sequencing method, using an Applied Biosystem 1310 automated system according to manufacturer's protocol using primers H52, H54, H59, H61, H63, H64, H31 (Table 1). All primers were designed using Primer3 software (Rozen and Skaletsky, 1998). The sequencing scheme is presented in Figure 1.

Table 1 Primers: sequences and uses. The mutant codon in HM3 is shown in bold

Primer	Sequence	Use
H52	5'-GACTCCATTATGCTGGTGGAA-3'	PCR, sequencing
H54	5'-CTATTGGCCATGTGGGGCCTG-3'	Sequencing
H53	5'-TCTCAGGGGATGGAGTGG-3'	PCR
H59	5'-ACAGATTTGTGAGGTCAGTGG-3'	Sequencing
H61	5'-AACCAGGGTTCCTTCTCCCT-3'	Sequencing
H63	5'-CGCAACTACGACCACATCCTC-3'	Sequencing
H64	5'-CCAGGATGGCTCTCCTGGC-3'	Sequencing
H31	5'-GTCTGTCCTCTTGAGCACGTG-3'	Sequencing
H34	5'-CTCCTTAAGTGCATACTTCAG-3'	PCR
H36	5'-GGGCTAGTTCTGTGGCTGAA-3'	PCR
HC5	5'-GGTGGTCATATGGTGGACAGCGTG-TACCGG-3'	pET-cloning
HC32	5'-AACATTGGTACCCTTGGCAAAGCA-TCAGTCTGTCCTCTTGAG-3'	pET-cloning
HM3	5'-GTGAGCGAAACT GCT TCCAAGGGC-ACATG-3'	Mutagenesis

Preparation of full-length cDNA carrying the mutation present in the patient, and insertion of this mutated DNA into an expression vector: Plasmid pGE3, containing wild-type human GNMT cDNA in the pET-17b vector for expression in *E. coli* (Luka and Wagner 2003), was used as starting material to prepare mutated human GNMT cDNA. Mutagenesis was carried out by a megaprimer method (Sarkar and Sommer 1990). As the potential 5' primer for preparation of full-sized cDNA, a 444 nucleotide megaprimer, MT1, was synthesized using HC5 (Table 1) as 5' primer and HM3 (Table 1) as 3' primer. The latter (reverse) primer contained a sequence that would lead to formation of the mutated nucleotide codon, AAC to AGC. To synthesize the megaprimer, 40 cycles of PCR reaction were carried out using denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1.5 min, followed by incubation at 72°C for 5 min.

The synthesized MT1 primer was then used as the 5' primer for preparation of full-size mutated cDNA (M3-cDNA) with HC32 as 3' primer. The 25 cycles of

**Figure 1** The scheme of sequencing of patient's GNMT gene. The relative position of primers from Table 1 used for PCR and sequencing is shown

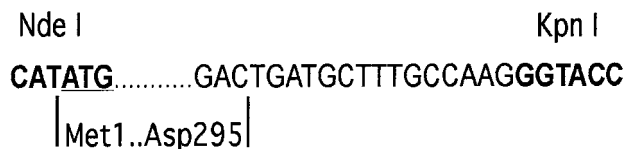


Figure 2 The position in the pET 17b expression vector of the patient's cloned GNMT cDNA. The first (ATG/Met 1) and the last (GAC/Asp 295) codons and amino acid residues of the GNMT cDNA are shown, separated by ellipses to indicate the remainder of the coding portions of the cDNA. The restriction sites for *NdeI* and *KpnI* are shown in bold

PCR reaction included denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 68°C for 2 min, followed by incubation at 68°C for 8 min.

Full-length mutated cDNA was cloned into pET-17b using *NdeI* and *KpnI* sites by standard cloning procedures to prepare a plasmid, pME3, for expression in *E. coli*. The pME3 plasmid DNA was sequenced with T7-universal primers and primers H52 and H63, and the DNA was shown to carry the mutation in the correct position (Figure 2).

Expression and analysis of mutant GNMT: Expression and purification of mutated human GNMT was done according to the protocol for expression and purification of wild-type human GNMT (Luka and Wagner 2003). A protein sample of at least 95% purity was prepared.

Enzyme assay: Enzyme activity was assayed by the discontinuous method described earlier (Wagner et al 1985). Reaction mixtures of 100 µl volume containing 200 µmol/L [³H]AdoMet, 200 mmol/L Tris-HCl, pH 7.5, 5 mmol/L DTT, 20 mmol/L glycine and 1–2 µg of GNMT were incubated for 15 min at 25°C. The reaction was terminated by addition of 50 µl of 10% TCA. Unreacted AdoMet was removed by addition of 250 µl of acid-treated charcoal suspended in 0.1 mol/L acetic acid (40 mg/ml). After cooling on ice for 15 min, charcoal was removed by centrifugation, and the radioactive reaction product, sarcosine, was counted in a scintillation counter (Tri-Carb Liquid Scintillation Analyzer (Packard Instrument Co., Meriden, CT, USA).

CASE HISTORY

The patient is an adopted male, born in 1998, the fourth offspring of parents of gypsy origin. He was referred to the University A' Department of Pediatrics of the Hippocraton General Hospital, Thessaloniki, Greece, in October 2002 because of persistently elevated serum aminotransferases of unknown aetiology. No information regarding pregnancy and delivery was available. His birth weight was 3350 g and his Apgar score was normal. Ten hours after birth he manifested projectile vomiting and refusal to feed. He was admitted to a paediatric clinic because of suspected septicaemia and was later moved to the ICU because of episodes of bradycardia. He was kept in an incubator for 2 days, after which he recovered completely. Laboratory investigation for infection was negative and he was discharged. At 9 months, when he was considered for adoption, physical examination was unremark-

able, development milestones were normal, and tests for hepatitis B or C viruses, HIV and inherited anaemias were negative. His blood group was B positive.

Mildly elevated serum alanine transaminase (ALT) and aspartate transaminase (AST) were first observed at age 2 years during a routine biochemical and haematological workup. There was no hepatomegaly. Levels of transaminases one month later were very close to the upper ends of the reference ranges. Two years later, at age 4 years, repeat testing for transaminases again showed increased levels. Haemoglobin, complete blood counts, glucose, blood urea nitrogen, electrolytes, albumin, alkaline phosphatase, γ -glutamyltransferase, creatine kinase, urea, creatinine, bilirubin, uric acid, amylase, prothrombin time, partial thromboplastin time, 24 h urinary copper, serum copper and ceruloplasmin were within the normal ranges. There was a mild elevation of ferritin to 173 ng/ml (normal values 13–150 ng/ml). Protein electrophoresis, C3, C4 antinuclear, anti-smooth-muscle antibodies, and liver/kidney microsomal antibodies were all normal. Serology tests for hepatitis C virus, cytomegalovirus, herpes simplex virus 1 and 2 were negative, whereas anti-hepatitis B surface antigen and anti-hepatitis A virus antibodies were positive owing to previous hepatitis B virus and hepatitis A virus immunizations, and testing for Epstein–Barr virus was positive for the viral capsid antigen and negative for nuclear capsid antigen. Whereas early samples for serum triglycerides and total cholesterol were completely normal, recent samples have shown a tendency to be increased: 155 mg/dl (reference range 40–144) and 248 mg/dl (reference range 120–220), respectively. Serum transaminases are followed up regularly and during a period of 3 years ranged from 31 to 154 U/L for aspartate transaminase (reference range 10–34) and from 34 to 180 U/L for alanine transaminase (reference range 7–33). Blood ammonia and lactate were normal. The results of analyses of urine organic acids, as well as quantitative plasma amino acid assays, were normal except for marked increases of methionine: 508–627 μ mol/L in three separate samples (reference range 8–40). Plasma tHcy was 9.4 μ mol/L and tyrosine was within the normal range. Urinary amino acids were normal except for an increased methionine excretion: 60 μ mol/mmol creatinine (reference range 5–21).

Liver ultrasound showed increased echogenicity of periportal spaces only. A needle biopsy of the liver at age 5 years was reported to show normal architecture of the hepatic parenchyma without significant morphological changes. Portal spaces were of normal width, cellularity, number and bile-duct morphology. A few hepatic cells showed mild hydropic degeneration. PAS staining for glycogen, Pas-d for α 1-anthrpsin granules, and orcein–Shikata for copper-binding protein granules were negative.

Unfortunately, no information could be obtained on the health history of the patient's biological family.

RESULTS

To further investigate the cause of the hypermethioninaemia, assays of metabolites related to methionine metabolism were performed at age almost 5 years (Table 2). Greatly elevated concentrations of plasma methionine persisted. At 11.1–12.1 μ mol/L, plasma tHcy was, at most, minimally elevated (reported reference ranges for young

Table 2 Concentrations of metabolites in plasma from the patient

Metabolite (units)	Concentration ^a		Reference range
	Sample 1	Sample 2	
Methionine (μmol/L)	1005	1049	13–45
AdoMet ^b (nmol/L)	2222	1643	93 ± 16
AdoHcy (nmol/L)	43	32	15–45
tHcy (μmol/L)	11.1	12.1	5.4–13.9 ^c
Cystathionine (nmol/L)	900	740	44–342
tCys (μmol/L)	217	206	203–369
Phosphatidylcholine (μmol/L)	2622	2641	1500–2559
Choline (μmol/L)	14.0	10.2	10.7–19.7
Betaine (μmol/L)	382	218	26–67
Dimethylglycine (μmol/L)	11.7	12.1	1.4–5.3
N-Methylglycine (μmol/L)	1.6	1.4	0.6–2.7

^aValues from two plasma samples obtained about 6 weeks apart are listed

^bAbbreviations: AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; tHcy, total homocysteine; tCys, total cysteine

^cThis reference range is that of the laboratory in which the tHcy assays were performed, and applies to subjects of ages 18–65 years. There is general agreement in the literature that plasma tHcy is lower in young children than in adults. Representative published values include the following: 5.8 μmol/L (range 3.3–8.3) at age 2 months–10 years (Vilaseca et al 1997); 8.9 μmol/L ± 0.9 (SD) for males 0.1–4.0 years of age (Reddy 1997); ~5 μmol/L for boys and girls before puberty (Schneede et al 2000); and 2.6–8.9 μmol/L at ages less than 10 years (Delvin et al 2000)

children range as high as 8.9 μmol/L, see footnote to Table 2). Plasma AdoMet was strikingly elevated, but N-methylglycine was normal. Together, these findings strongly indicated a diagnosis of GNMT deficiency. This possibility was then confirmed by molecular genetic identification of the presence of a mutation in the gene encoding GNMT, as described in the next sections.

Screening for mutations: The GNMT gene was screened for mutations using an automated sequencing system. Fragments of all exons and most of the introns, as well as about 300 nucleotides of the 5' and 3' flanking areas, were sequenced. In this paper we use the nucleotide numbering for the GNMT sequence specified by Chen and colleagues (see Gene Bank accession number GI 8671581) in which an adenine nucleotide in the first codon is numbered 1334 and the last cytidine nucleotide in exon 6 is numbered 4270. The coding sequence of the patient's GNMT gene was found to be identical to the published sequence (Chen et al 2000) with the exception of a single nucleotide substitution in an AAC codon of exon 3: 3415A>G. This would change asparagine 140 to serine (Figure 3). That this change is not a common polymorphism is shown by the fact that it was not observed in the GNMT coding regions of the almost 300 individuals subjected to polymorphism analysis by Tseng and colleagues (2003), or in those of an additional 10 control subjects studied in our laboratory (Luka and Wagner, unpublished observations). At the position of a previously identified 1289C>T polymorphism in which T is the more common nucleotide (Tseng et al 2003; Luka and Wagner, unpublished observations) the nucleotide in the present patient was C only.

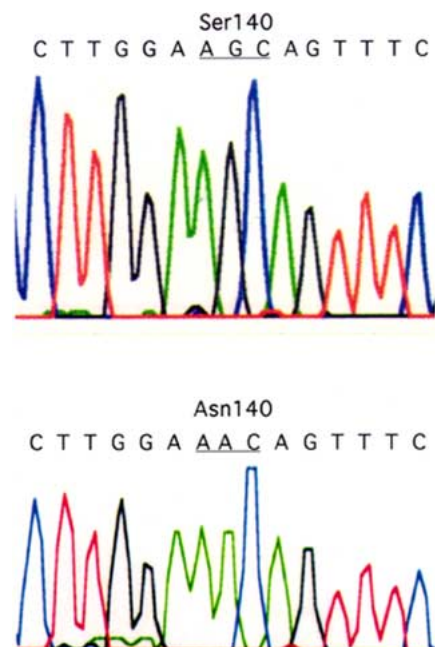


Figure 3 Nucleotide sequences of fragments of exon 3 of the GNMT gene are shown, with the wild-type sequence at the bottom and the sequence from the patient at the top. The codon containing the AAC to AGC mutation is underlined

Expression of the enzyme containing the Asn140Ser mutation and assay of its activity: Mutant human GNMT containing the Asn140Ser substitution was expressed in *E. coli* and the protein product was purified to virtual homogeneity. Using our standard incubation time and temperature (15–20 min, 25°C), with either the standard or increased concentrations of the substrates, no activity was detected. Only after a longer incubation (60 min) at a higher temperature (30°C) was a measurable amount of the reaction product, sarcosine, detected. Under these conditions the activity was so low (less than 1.0% compared to wild-type GNMT expressed and purified in the same manner) that it was not possible to determine the kinetic parameters of the mutant enzyme.

Concentrations of additional metabolites (Table 2): Plasma AdoHcy, tHcy and choline were within the reference ranges. Cystathionine was moderately elevated; phosphatidylcholine marginally so. Betaine and dimethylglycine were definitely elevated. These results are discussed below.

DISCUSSION

Diagnosis: The patient reported here presented with findings characteristic of what has been termed ‘isolated persistent hypermethioninaemia’ (Mudd et al 1995b):

elevated plasma methionine that has lasted in this case to age 5 years; normal plasma tyrosine and an absence of urinary succinylacetone that ruled out tyrosinaemia type I; liver disease not severe enough to explain the methionine elevation (Mudd et al 2001a); and tHcy elevated so slightly as to rule out cystathionine β -synthase deficiency (Mudd et al 2001b). Most such individuals for whom definitive diagnoses have been obtained have been shown to have deficient activities of methionine adenosyltransferase I/III (MAT I/III) (Mudd et al 2001b). That this is not so for the present patient was shown by his elevated plasma AdoMet, a finding not present in MAT I/III deficiency (Mudd et al 2000, 2001b). Very recently, an additional cause of 'isolated persistent hypermethioninaemia' has been identified, namely AdoHcy hydrolase deficiency. In addition to hypermethioninaemia, the only known patient with this deficiency had striking elevations of plasma AdoHcy and AdoMet (Baric et al 2003). The normal plasma AdoHcy of the present patient therefore rules out AdoHcy hydrolase deficiency. Fortunately, correct diagnosis for our patient was facilitated by the lack of elevation of plasma sarcosine in the presence of elevated AdoMet, a finding that strongly indicated he has GNMT deficiency (Mudd et al 2001a).

The mutation in the gene that encodes GNMT: Definitive proof of GNMT deficiency was provided by molecular studies of the patient's GNMT gene that showed he has a nucleotide change, 3415A>G in codon 3 that causes an Asn140Ser substitution. This substitution was shown not to be a common polymorphism, and expressed GNMT containing it had no more than a trace of GNMT activity. No additional point mutation was found in the coding regions of the patient's GNMT gene. Based on these results, one may conclude either that he is homozygous for this mutation or that he has a deletion on the other allele that overlaps the 3415A>G change and prevented PCR amplification of the region in question (Zschocke et al 1999). In either case, he would be expected to have, at most, only minimal residual GNMT activity. A protein structure for rat GNMT complexed with AdoMet and acetate (a competitive inhibitor of glycine) is reported in the Protein Database (Takata and Takusagawa 2003). This structure shows that Asn138 in the rat GNMT (the equivalent of Asn140 in the human enzyme) makes a hydrogen bond with acetate in the active site. Therefore, the Asn140Ser mutation in the present patient may be expected to markedly affect the binding of glycine at the active site of his GNMT, thereby explaining the profound effect of this mutation on his activity.

Other metabolites related to methionine metabolism (Table 2): Although formation of AdoHcy is to some extent impeded in the patient by deficient GNMT activity, the fact that normal concentrations of plasma AdoHcy were observed indicates that methyltransferases other than GNMT must be continuing to convert AdoMet to AdoHcy (perhaps aided by elevated concentrations of AdoMet) at a combined rate sufficient to maintain AdoHcy. Mammals have at least 38 such additional AdoMet-dependent methyltransferases (Clarke and Banfield 2001). The AdoHcy is then converted to homocysteine. Conversion of homocysteine to methionine is likely to be impeded by lack of methyltetrahydrofolate due to inhibition by AdoMet of methylenetetrahydrofolate reductase (Jencks and Matthews 1991; Kutzback and

Stokstad 1967; Matthews and Daubner 1982), but conversion to cystathionine is stimulated by AdoMet activation of cystathionine β -synthase (Finkelstein et al 1975; Kluijtmans et al 1996; Oliveriusova et al 2002). The net result is maintenance of a normal plasma tHcy level, perhaps with a tendency to an increased rate of cystathionine formation. The concentration of the latter compound would be further enhanced by inhibition by methionine of cystathionine gamma-lyase, the enzyme that cleaves cystathionine to cysteine (Stabler et al 2002). Together, these effects lead to a plasma tCys near the lower end of the reference range.

The slightly elevated concentrations of plasma phosphatidylcholine suggest that synthesis of this compound may be occurring at a faster than usual rate due to the increased AdoMet, the co-substrate for AdoMet-dependent phosphatidylethanolamine methyltransferase. This effect might account for the elevations of betaine and dimethylglycine, formed ultimately from phosphatidylcholine by way of free choline. The failure of plasma choline itself to be elevated does not support this hypothesis, but, in our opinion, given the uncertainties about the relationships between the cellular and the plasma concentrations of the compounds involved, and the difficulties of judging fluxes based on the concentrations of metabolic intermediates, neither does it negate the hypothesis. An additional contribution to the elevation of betaine (but not to that of dimethylglycine) may be due to the enhanced ability of cystathionine β -synthase activated by AdoMet (discussed above) to compete with betaine-homocysteine methyltransferase for any available homocysteine, thereby decreasing the rate of betaine removal.

Comparison of the present patient with the two previously reported: The deficit in GNMT activity in the present patient might be expected to be somewhat more severe than those in the two previous known individuals with this deficiency since they are each compound heterozygotes for two mutations that, when expressed individually, had 10–50% of wild-type activity (Luka and Wagner, unpublished observations), whereas this patient's expressed mutant enzyme had scarcely detectable activity. We note, however, that predictions of *in vivo* GNMT activities on the basis of such expressed activities are difficult, given that GNMT normally occurs as a tetramer of identical subunits (Ogawa and Fujioka 1982) and that the effects of the mutations in question on subunit assembly and interactions are presently not known.

A possible means to judge the relative severities of the GNMT deficits in these patients might be to compare their elevations of plasma methionine and AdoMet. However, any such comparisons may be complicated by the effects of dietary methionine and (perhaps) development. In the younger previous patient, at age 2.4 years plasma methionine and AdoMet were both normal when her dietary intake of methionine was restricted to a mean of 30 mg/kg per day. At age 2.4–3.9 years, on her normal diet that provided a mean methionine intake of 81 mg/kg per day, plasma methionine ranged from 483 to 711 μ mol/L and plasma AdoMet from 1149 to 3878 nmol/L. Her older brother at age 9.6 years on a normal diet containing 48 mg methionine/kg per day had a plasma methionine concentration of 426 μ mol/L and an AdoMet of 2252 nmol/L (Mudd et al 2001). The present patient at age almost 5 years while on a normal diet estimated to provide 58 mg methionine/kg per day had

higher plasma methionine values of 1005 and 1048 $\mu\text{mol/L}$. His plasma AdoMet elevations (Table 2) were more or less comparable to those found in the previous patients. It appears that, at best, the concentrations of these metabolites in plasma provide imperfect criteria whereby to judge the severity of the deficit in GNMT activity.

It is noteworthy that, in so far as assayed in the former patients, methionine-related metabolites in addition to those just discussed followed the same trends as noted above for the present patient. On normal diets, plasma AdoHcy and tHcy were normal (Mudd et al 2001), cystathionine and dimethylglycine were elevated, and tCys was at the low end of the reference range.

Clinically, the present patient is similar to the previous two in having mild but persistent elevations of serum transaminases, but no other serious clinical abnormalities. Whereas the earlier two both had hepatomegaly and, in the girl, histological evidence of mild centrilobular fibrosis, neither of these features was observed in the present patient. Nevertheless, in view of the fact that many alternative causes of liver abnormalities have been ruled out for each of the three, the presence of mild hepatitis-like elevations of transaminases in all of them significantly strengthens the likelihood that there is a causative relationship between these manifestations and the GNMT deficiency. It was previously suggested that impairment of GNMT activity, by limiting the amount of homocysteine available for transsulphuration to cysteine, might lead to liver problems because a decreased rate of cysteine synthesis might in turn limit the rate of formation of glutathione (Mudd et al 2001a). This appears to remain a possibility.

Important remaining questions about GNMT deficiency: The studies carried out to date with the three known GNMT-deficient individuals leave significant questions unanswered.

What is the long-term prognosis? Because the oldest patient was only 9.7 years of age at last report (Mudd et al 2001), it is possible that additional adverse effects, or a worsening of the existing ones, will develop in the future. Among these are possible progression of the hepatitis-like abnormalities to more severe liver damage with cirrhosis. Other possibilities include damage to additional organs. GNMT activity is expressed not only in liver but also in cells of renal proximal convoluted tubules, pancreatic acinar tissue, submaxillary gland striated duct and jejunal villi epithelium (Yeo and Wagner 1994). GNMT messenger RNA has been reported also in prostate of the rat (Chen et al 2000). Careful attention to these organs and their functioning may be indicated in GNMT-deficient patients.

Are additional cases of GNMT deficiency going undiagnosed and, if so, how might they be looked for? The two *propositi* among the known cases of GNMT deficiency were each investigated further because of unexplained elevations of methionine and serum transaminases. Abnormally high plasma AdoMet levels and normal sarcosines were then found, leading to molecular genetic studies that proved GNMT deficiency. It might be useful to assay plasma AdoMet and sarcosine more routinely in individuals with elevated serum transaminases and hypermethioninaemia, especially if the transaminase elevations are mild and the methionine elevation

relatively high. However, limitation of such assays to patients with both hypermethioninaemia and elevated serum transaminases might introduce ascertainment bias. Some GNMT-deficient individuals, especially those with milder activity deficits, may turn out to have hypermethioninaemia, but not elevations of transaminases.

An additional tactic would be to search among infants found on newborn screening to have elevated blood methionine concentrations. Routine screening for this abnormality is at present carried out in 17 states in the United States and in a number of other countries to detect the elevated methionine that accompanies homocystinuria due to cystathionine β -synthase deficiency (Mudd et al 1995a; National Newborn Screening Report – 1999). The cut-off points to indicate abnormal methionine elevations currently in use in many such programmes range from close to 67 $\mu\text{mol/L}$ to as high as 268 $\mu\text{mol/L}$ (see, for example, Peterschmitt et al 1999 and the National Newborn Screening Report – 1999). An example of the frequency with which elevated blood methionine concentrations are found is provided by the recent experience of the New England Newborn Screening Program: using a cut-off point of 67 $\mu\text{mol/L}$ for methionine, 71 infants were flagged in a total population of 257 000 infants screened. Of these, 53 were classified as NICU/VLBW (i.e. neonatal intensive-care unit/very low birth weight) (Zytokovitch et al 2001). If the elevated methionine persists and plasma tHcy is found not to be markedly elevated in an infant with such an extent of hypermethioninaemia, other causes of this abnormality might be considered. GNMT activity is very low in fetal liver of experimental animals during gestation, increasing somewhat at times close to birth, and much more markedly in the neonatal period (Cook and Wagner 1981; Heady and Kerr 1975). Similar studies in humans have not been reported. Thus, it is uncertain how soon after birth GNMT deficiency will manifest itself by elevation of blood methionine above the cut-off points in question. A direct answer is not available because none of the patients in the three known cases of GNMT deficiency were screened for blood methionine as infants. Nevertheless, hypermethioninaemia in an infant may be due to a variety of causes other than cystathionine β -synthase deficiency. These include tyrosinaemia type I, increased dietary intake of methionine (Mudd et al 2003), and MAT I/III deficiency (Mudd et al 2001b). Addition to this list of GNMT deficiency may turn out to be merited, and even *S*-adenosylhomocysteine hydrolase deficiency is a possibility, although newborn blood methionine was not abnormal in the one known case of this disease (Baric et al 2003). Thus in cases of unexplained infant hypermethioninaemia it may be opportune to utilize analytical tools that have become available recently, such as assay of plasma AdoMet, AdoHcy, cystathionine and sarcosine. These assays facilitate distinction between the several causes of hypermethioninaemia.

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