Mutations in genes encoding the glycine cleavage system predispose to neural tube defects in mice and humans

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Neural tube defects (NTDs), including spina bifida and anencephaly, are common birth defects of the central nervous system. The complex multigenic causation of human NTDs, together with the large number of possible candidate genes, has hampered efforts to delineate their molecular basis. Function of folate one-carbon metabolism (FOCM) has been implicated as a key determinant of susceptibility to NTDs. The glycine cleavage system (GCS) is a multi-enzyme component of mitochondrial folate metabolism, and GCS-encoding genes therefore represent candidates for involvement in NTDs. To investigate this possibility, we sequenced the coding regions of the GCS genes: AMT, GCSH and GLDC in NTD patients and controls. Two unique non-synonymous changes were identified in the AMT gene that were absent from controls. We also identified a splice acceptor site mutation and five different non-synonymous variants in GLDC, which were found to significantly impair enzymatic activity and represent putative causative mutations. In order to functionally test the requirement for GCS activity in neural tube closure, we generated mice that lack GCS activity, through mutation of AMT. Homozygous Amt⁻/⁻ mice developed NTDs at high frequency. Although these NTDs were not preventable by supplemental folic acid, there was a partial rescue by methionine. Overall, our findings suggest that loss-of-function mutations in GCS genes predispose to NTDs in mice and humans. These data highlight the importance of adequate function of mitochondrial folate metabolism in neural tube closure.

INTRODUCTION

Neural tube defects (NTDs), such as spina bifida and anencephaly, are common birth defects that result from failure of closure of the neural folds during embryonic development (1). Although NTDs are among the commonest birth defects in humans, the causes are still not well understood. This is most likely due to their complex, multifactorial causation

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which is thought to involve contributions from both genetic and environmental factors (2–4). The potential complexity of NTD genetics is illustrated by the fact that more than 200 different genes give rise to NTDs when mutated in mice (5,6). Moreover, inheritance patterns in humans suggest a multifaceted model in which an affected individual may carry two or more risk alleles, which by themselves may be insufficient to cause NTDs (2).

Folate one-carbon metabolism (FOCM) is strongly implicated as a determinant of susceptibility to NTDs since suboptimal maternal folate status and/or elevated homocysteine are established risk factors, whereas periconceptional maternal folic acid supplementation can reduce the occurrence and recurrence of NTDs (7,8). Nevertheless, the precise mechanism by which folate status influences NTD risk remains elusive (7,9). FOCM comprises a network of enzymatic reactions required for synthesis of purines and thymidylate for DNA synthesis, and methionine, which is required for methylation of biomolecules (Fig. 1A) (9). In addition to the cytosol, FOCM also operates in mitochondria, supplying extra one-carbon units to the cytosolic FOCM as formate (Fig. 1A) (10).

Genes that are functionally related to folate metabolism have been subjected to intensive genetic analysis in relation to NTD causation, principally through association studies (reviewed in 3,4,11). In the most extensively studied gene, MTHFR, the c.677C>T SNP is associated with NTDs in some, but not all, populations. However, other FOCM-related genes have largely shown non-significant or only mild associations. Given the apparently complex inheritance of the majority of human NTDs, many association studies have been hampered by limitations on sample size. Moreover, although positive associations have been noted for other genes including DHFR, MTHFD1, MTRR and TYMS (12,13), these have not been replicated in all populations, and additional studies are required. The hypothesis that genetically determined abnormalities of folate metabolism may contribute to NTD susceptibility is supported by the observation of defects of thymidylate biosynthesis in a proportion of primary cell lines derived from NTDs (14). However, these defects do not correspond with known polymorphisms in FOCM-related genes. Overall, it appears likely that genetic influences on folate metabolism remain to be identified in many NTDs.

A potential link between mitochondrial FOCM and NTDs was suggested by the finding of an association of increased NTD risk with an intronic polymorphism in MTHFDI (15). Another component of mitochondrial FOCM, the glycine cleavage system (GCS), acts to break down glycine to carbon units to THF, generating 5,10-methylene-THF from THF. Genes encoding enzymes for each reaction are indicated in italics. DHF, dihydrofolate; THF, tetrahydrofolate. (B) Summary of the GCS. The glycine cleavage reaction is catalysed by the sequential action of four individual enzymes: GLDC, GCSH, AMT and DLD. The first three of these (shaded grey) are specific to the GCS. Glycine is broken down into CO₂ and NH₃, and donates a one-carbon unit (indicated in bold) to THF, generating 5,10-methylene-THF. The other carbon in glycine (indicated in italics) enters CO₂.

have been found to be abundantly expressed in the neuroepithelium during embryogenesis in the rat (20).

We hypothesized that modulation of GCS activity has the potential to influence efficacy of cellular FOCM during the period of neural tube closure and, hence, susceptibility to NTDs. Therefore, in the current study, we screened genes encoding GCS components for possible mutations in NTD patients and controls. We tested variant proteins for loss of function by enzymatic assay and mice lacking GCS function were generated, to test the effect on embryonic development.

RESULTS

The hypothesis that genes of the GCS represent candidates for involvement in NTDs prompted us to screen for potential mutations in patient samples. Coding exons of AMT (9 exons), GCSH (5 exons) and GLDC (25 exons) were sequenced in a total of 258 NTD patients comprising cohorts from Japan, the UK and Sweden. Each of the major categories of NTDs was represented among study samples, including anencephaly (n = 38), spina bifida (n = 198) and craniorachischisis (n = 22).
In *AMT*, we identified two novel sequence variants predicted to result in non-synonymous missense changes, c.589G>C (D197H) and c.850G>C (V284L), in anencephaly and spina bifida patients, respectively, from the UK cohort (Table 1). Neither variant was present in 526 UK or 36 Japanese control subjects or in the SNP databases dbSNP and 1000 Genomes. An additional missense variant, E211K, was also identified in three spina bifida patients, two from the UK and one from Sweden. Causative mutations in *AMT* have been found previously in an autosomal recessive inborn error of metabolism, non-ketotic hyperglycaemia (NKH) (17). The E211K variant had previously been identified in an NKH family but was established as likely to be a non-functional polymorphism by segregation (21). Therefore, this variant is considered unlikely to be causally related to NTDs.

Exon sequencing of *GCSH* revealed eight single-base substitutions, one of which (c.53C>T, p.A18V) was a novel change found in both an NTD and a single control (Table 1). The others all corresponded to known SNPs, which did not suggest a role for *GCSH* in NTDs.

Next we turned our attention to *GLDC*, in which we identified 27 single-base substitutions (Table 1), including 11 silent nucleotide changes, 15 non-synonymous changes and a splicing acceptor variant of intron 19 (c.2316-1G>T) and c.1705G>A in *GLDC*, whereas no other individuals carried more than one of the nucleotide changes listed here. Eight silent polymorphisms and four missense variants present in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) are not listed in this table and include: *AMT*: c.954G>A (p.R318R, rs11715915); *GLDC*: c.249G>A (p.G83G, rs12341698), c.438G>A (p.T146T, rs13289273), c.501G>A (p.E167E, rs13289273), c.668C>T (p.L220L, rs2228095), c.666T>C (p.E211K, rs22004164), c.671G>C (p.D222D, rs28617412) and c.683G>C (p.L229F, rs28617412), and for *GCSH*: c.623C>T (p.A18V), c.660C>T (p.G18C), c.1525C>T (p.A208D), c.1570G>C (p.D197H) and c.2965A>T (p.L916L). Grey shading indicates loss-of-function mutations, based on enzymatic activity in the *in vitro* expression study or splicing defect.

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### Table 1. Nucleotide changes in NTD patients and controls identified by exon sequencing of AMT, GLDC and GCSH

| Location | Nucleotide change | Effect | Number of mutation carriers in UK cohorts (type) | Number of mutation carriers in the Japanese cohort (type) | Number of mutation carriers in the Swedish cohort (type) | Variant GLDC enzyme activity |
|----------|------------------|--------|-----------------------------------------------|-------------------------------------------------|-------------------------------------------------|---------------------------------|
| **AMT**  |                  |        | (NTD group) (n=166) | (Control group) (n=189) | (NTD group) (n=14) | (Control group) (n=36) | (NTD group) (n=145) | (Control group) (n=145) |
| Exon 2   | c.103A>C         | p.R35R | 0 | 1 | 0 | 0 | 0 | — |
| Exon 6   | c.214A>G         | p.T72A | 0 | 0 | 0 | 0 | 1 | 0 |
| Exon 7   | c.825T>A         | p.N275K | 0 | 0 | 0 | 0 | 0 | 0 |
| Exon 1   | c.52G>T          | p.G18C | 0 | 0 | 0 | 0 | 2 (SBA) | 2 |
| Exon 12  | c.1508A>C        | p.E503A | 0 | 0 | 0 | 0 | 0 | 0 |
| Exon 14  | c.1953T>C        | p.H651H | 0 | 0 | 0 | 0 | 0 | 0 |
| Exon 19  | c.2203G>T        | p.V735I | 0 | 0 | 0 | 0 | 2 | 0 |
| Intron 19|                  |        | 0 | 0 | 0 | 0 | 0 | 0 |
| Exon 20  | c.2380G>A        | p.A794T | 0 | 0 | 0 | 0 | 2 (SBA) | 2 |
| Exon 21  | c.2474G>A        | p.G825D | 0 | 0 | 0 | 0 | 1 (An) | 0 |
| Exon 22  | c.2487C>T        | p.A829A | 0 | 0 | 0 | 0 | 0 | 0 |
| Exon 23  | c.2565A>C        | p.A855A | 0 | 0 | 0 | 0 | 0 | 0 |
| Exon 24  | c.2964G>A        | p.R988R | 0 | 0 | 0 | 0 | 0 | 0 |
| Exon 1   | c.53C>T          | p.A18V | 1 (An) | 0 | 0 | 0 | 0 | 0 |
| **GCSH** |                  |        |                                                                                                       |

All nucleotide changes were found in heterozygous form. One individual carried c.52G>T and c.1705G>A in *GLDC*, whereas no other individuals carried more than one of the nucleotide changes listed here. Eight silent polymorphisms and four missense variants present in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) are not listed in this table and include: *AMT*: c.954G>A (p.R318R, rs11715915); *GLDC*: c.249G>A (p.G83G, rs12341698), c.438G>A (p.T146T, rs13289273), c.501G>A (p.E167E, rs13289273), c.668C>T (p.L220L, rs2228095), c.666T>C (p.E211K, rs22004164), c.671G>C (p.D222D, rs28617412) and c.683G>C (p.L229F, rs28617412), and for *GCSH*: c.623C>T (p.A18V), c.660C>T (p.G18C), c.1525C>T (p.A208D), c.1570G>C (p.D197H) and c.2965A>T (p.L916L). Grey shading indicates loss-of-function mutations, based on enzymatic activity in the *in vitro* expression study or splicing defect.

*Residual enzymatic activity of GLDC mutant protein is expressed as %activity of the wild-type enzyme (Fig. 2).*

*SBA, spina bifida aperta; SBO, spina bifida occulta; An, anencephaly; Crn, craniorchiasisichis.*

*Total number of UK, Japanese or Swedish NTD patients.*

*This variant was previously established as likely to be a non-functional polymorphism by segregation in an NKH family (21).*

*A biochemical test of folate metabolism, the dU suppression test, was previously performed on primary fibroblasts derived from this patient and showed a defect of thymidylate biosynthesis to be present (14).*

*p.A569T has previously been reported as a pathogenic mutation in a patient with typical NKH (21).*
enzymatic function. In the case of G18C and A794T, which occurred in both NTDs and controls, there was no significant loss of enzymatic activity, suggesting that these are unlikely to be causative mutations.

Having identified putative mutations in AMT and GLDC in NTD patients, we hypothesized that loss of GCS function could predispose to development of NTDs. In order to directly test the functional requirement for GCS activity in neural tube closure, we generated mice that lacked GCS activity, using a gene trap (OmniBank, OST181110) of the AMT gene. The vector was located in intron 2, resulting in a truncated transcript that lacked exons 3–9 (Fig. 3). The efficacy of the gene-trap vector in generating chimeras, and germ-line transmission. LTR, long terminal repeats; SA, splicing acceptor site; Neo, neomycin phosphotransferase gene; pA, polyadenylation sequence. (B) For genotyping, mouse genomic DNA was subjected to allele-specific amplification with F, R1 and R2 primers (Supplementary Material, Table S1). A genomic fragment of 320 bp was amplified from the AMT-mutant allele. (C) RT-PCR analysis of Amt mRNA expressed in the brain and liver of Amt-mutant mice. Primers in exon 1–2 generated a 121 bp band irrespective of mouse genotypes. RT-PCR in which either one (f2-r2) or both (f3-r3) primers were located in exons 3 resulted in two bands, 220 and 355 bp cDNA fragments, respectively, in Amt+/+ and Amt−/− mice, but not in Amt−/−. The Amt mRNA in mice carrying the trap vector was, therefore, aberrantly spliced at the end of exon 2, resulting in truncation of Amt mRNA in Amt−/− mice.
\[ \text{Table S2). Six were instances of maternal transmission and one involved paternal transmission. We hypothesize that absence of an overt NTD phenotype in parents who carry a deficient GLDC allele may result from incomplete penetrance, or lack of additional genetic or environmental factors which are predicted to be necessary for NTDs owing to their multifactorial aetiology. We also note that partial penetrance is a feature of numerous mouse models of NTDs (5,8).} \]

\[ \text{Inherited GCS deficiency, owing to mutation of AMT and/or GLDC, has been shown to cause NKH in humans (17). NKH is a rare, autosomal recessive, inborn error of metabolism, characterized by accumulation of glycine and encephalopathy-like neurological signs, including coma and convulsive seizures in neonates. GCS activity is greatly diminished in NKH patients and they would, therefore, be predicted to be at increased risk of NTDs. It is possible that NTDs may occur in combination with NKH but as anencephaly is a lethal condition, co-existing challenge owing to their multigenic inheritance and the potential influence of environmental factors, either predisposing or ameliorating. Several lines of evidence indicate a requirement for FOCM in neural tube closure and, therefore, GCS-encoding genes provide excellent candidates for possible involvement in NTD susceptibility.} \]

\[ \text{We identified putative mutations in AMT and GLDC which include a splice acceptor mutation and a number of non-synonymous variants that were absent from a large group of population-matched controls, as well as from public SNP databases. In the case of GLDC, enzymatic assay confirmed that several mutations resulted in significant loss of enzyme activity. Finally, in vivo functional evidence of a requirement for GCS function in neural tube closure was provided by the occurrence of NTDs in AMT−/− mice lacking GCS activity. Together these findings indicate that mutations in GLDC and AMT predispose to NTDs in both mice and humans.} \]

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NKH would go undetected. Lack of NTDs in NKH patients may also reflect the multigenic nature of NTDs, which require the presence of additional risk alleles in non-GCS genes. NKH is a relatively rare condition, with a prevalence of 1/63 000 births in British Columbia (24) and 1/250 000 in the USA (25). It is therefore possible that an increased risk of NTDs among carriers of GCS mutations in NKH families may not have been noted and this possibility is worthy of investigation. Based on estimated carrier frequency and the incidence of mutations among NTD patients, we predict that NTDs might be expected among 1/150 of the siblings of NKH patients (see Supplementary Material, Table S3 for estimation calculation). One case report of an NKH patient with a GLDC mutation describes the additional presence of spinal cord hydromyelia (19). This condition is often associated with low spinal defects (involving secondary neurulation), but it is also possible that the expanded spinal canal was also present at a higher level and might indicate a limited defect in primary neurulation.

The mutations described in the current study were all present in heterozygous form and, therefore, are hypothesized to be insufficient to cause NKH while predisposing to NTDs. For example, in the current study we found four NTD patients and one control individual to be heterozygous for the A569T mutation, which is shown to result in reduced enzyme activity. This mutation was previously identified in a Caucasian patient with typical NKH, in combination with a second mutation, P765S (26), confirming that it is deleterious in vivo. Hence, we predict that, depending on the co-existing genetic milieu, the A569T variant may cause NKH, predispose to NTDs or be compatible with normal development.

The high incidence of NTDs in AMT mutant mice is particularly notable as NTDs have not previously been found to be a common feature of mouse models deficient for folate-metabolizing enzymes. This includes null mutants that have been reported for eight other genes that encode enzymes in FOCM (Fig. 1A) (27). Four have normal morphology at birth (Cbs, Mthfd1, Mthfr and Shmt1) (28–31), Mthfd2 null embryos die by E15.5 but neural tube closure is complete (32) and null mutants for Mtr, Mtrr and Mthfs die before E9.5, prior to neural tube closure (33–35). Although analysis of mouse mutants has not supported a role for single-gene mutations in FOCM as major causes of NTDs, a requirement for cellular uptake of folate for neural tube closure has been demonstrated in Foh1 null embryos, in which NTDs occur when rescued from early lethality by folic acid supplementation (36). There is also considerable evidence for possible involvement of gene–environment and/or gene–gene interactions in NTDs. For example, in Pax3 mutant (splotch) embryos, which exhibit a defect of thymidylate biosynthesis, dietary folate-deficiency increases the frequency of cranial NTDs (23,37). Similarly, a diet deficient in folate and choline causes NTDs in Shmt1 mutant embryos, whereas Shmt1 and Pax3 mutations exhibit genetic interaction (38).

Regarding the mechanisms by which GCS mutations affect neural tube closure, a key question is whether NTDs are caused by impairment of FOCM or by another cause such as glycine accumulation. Modelling of hepatic FOCM, based on biochemical properties of folate-metabolizing enzymes (39), predicts that loss of the mitochondrial GCS reaction would reduce the efflux rate of formate to the cytosol by ~50%. This results in reduced synthesis of purines and thymidylate, which are essential for the rapid cell division in the closing neural folds. Interestingly, a UK patient with anencephaly who was found to carry the GLDC loss-of-function mutation P509A in the current study (Table 1) was previously found to have impaired thymidylate biosynthesis, assayed in cultured fibroblasts (14). These findings support the hypothetical link between diminished GLDC function, reduced thymidylate biosynthesis and development of NTDs. Reduced thymidylate biosynthesis and diminished cellular proliferation are proposed to underlie folate-related cranial NTDs in splotch (Pax3) mouse mutants (37,38).

As well as impairment of nucleotide biosynthesis, the predicted effect of diminished GCS activity in reducing production of methionine (39) may also be of relevance as methionine is the precursor for the methyl donor S-adenosylmethionine. Indeed, metabolic tracing experiments suggest ~80% of 1C units in the methylation cycle are generated within mitochondrial FOCM (40). Impairment of the methylation cycle and/or DNA methylation is known to cause NTDs in mice (41) and is proposed as a possible cause of human NTDs (7,42). It was therefore notable that we found a preventive effect of methionine supplementation in Amt−/− mice. Together, these findings suggest that FOCM, required for both thymidylate biosynthesis and methylation reactions that are essential for neural tube closure, may be functionally deficient in individuals who have mutations in GLDC or AMT.

**MATERIALS AND METHODS**

**Patient cohorts and sequencing**

Mutation analysis by DNA sequencing was performed on all exons of AMT, GCSH and GLDC as described (26). Cases comprised Japanese patients with anencephaly (n = 14) and two separate cohorts of UK patients with a diagnosis of anencephaly (combined n = 24), spina bifida (n = 122) or craniorachischisis (n = 22). In addition, the exons of AMT, GCSH and GLDC were sequenced in 76 Swedish patients with spina bifida. Unaffected controls, completely sequenced for these genes, comprised 36 Japanese and 189 unrelated UK subjects. Exons found to contain missense mutations were also sequenced in a further cohort of 192 well-characterized UK controls (43) and in 145 Swedish controls. This study was approved by the Ethical Committees of Tohoku University School of Medicine, UCL Institute of Child Health, Newcastle University and the Karolinska Institute.

**Enzymatic assay of GCS activity and GLDC activity**

GCS activity was measured in mouse liver samples by a decarboxylation reaction using [1,14C]glycine as described (22). For analysis of GLDC activity, wild-type and mutant GLDC cDNAs were cloned into pCAG expression vector, kindly provided by Professor Jun-ichi Miyazaki (Osaka University, Japan) (44). Constructs were transfected into COS7 cells, which were harvested as described previously and cell pellets stored at −80°C prior to analysis (45). GLDC
enzymatic activity was determined, in triplicate, by exchange reaction between carbon dioxide and glycine using NaH14CO3 in the presence of excess recombinant bovine GCSH protein as described (22). An expression system of lipoylated bovine GCSH protein in *Escherichia coli* was kindly provided by Dr Kazuko Fujiwara (Tokushima University, Japan) (46). Statistical analysis was performed using SPSS software version 11.0 (SPSS, Inc., Chicago, IL, USA).

**Knockout of Amt by insertion of a gene-trap vector**

Mice carrying a gene-trap allele of *Amt* (here denoted *Amt−/−*) were generated at Lexicon Genetics, Inc. (Houston, TX, USA) using the OST181110 ES cell line. The genomic insertion site of the gene-trap vector was determined by inverse PCR and localized to intron 2 (Supplementary Material, Fig. S1). Total RNA was prepared from the mouse liver and brain at E18 for RT-PCR analysis (Supplementary Material, Fig. S1 and Table S1). *Amt−/−* mice were backcrossed with wild-type C57BL/6 mice for nine generations to generate a congenic line of mice on the C57BL/6 background, for use in biochemical and histological analyses. This study was approved by the Animal Experiment Committee of Tohoku University.

**Maternal supplementation with folic acid and related metabolites**

Dams were treated with folic acid (25 mg/kg), thymidine-1-phosphate (TMP; 30 mg/kg) or L-methionine (70 mg/kg) by intra-peritoneal injection, 2 h prior to mating and daily from E7.5–10.5. Doses were based on previous studies (23,47,48).

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

**Conflict of Interest statement.** None declared.

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