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**Focal Segmental Glomerulosclerosis in Patients With Complete Deletion of One
WT1 Allele**

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ORIGINAL ARTICLE

Attitudes toward non-invasive prenatal diagnosis among pregnant women and health professionals in Japan

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ABSTRACT

Objective This study aims to assess the attitudes toward non-invasive prenatal diagnosis (NIPD) and NIPD problems in clinical practice in Japan.

Methods A mail-in survey using a self-reported questionnaire was conducted among pregnant women and health professionals. The questionnaire enquired about attitudes, concerns, and expectations regarding NIPD.

Results The responses from 252 respondents revealed that pregnant women have more positive attitudes toward NIPD than health professionals. In addition, there were wide discrepancies in concerns and expectations about NIPD, between medical professionals and pregnant women. The respondents with less NIPD knowledge had a more positive attitude toward the clinical application of NIPD. There was concern expressed by clinical geneticists whether an NIPD test should be performed or not when there is a lack of knowledge about the NIPD. All of the health professionals emphasized the importance of providing genetic counseling prior to and after the testing.

Conclusion Pregnant women place importance on the safety and non-invasiveness of the NIPD tests, whereas medical professionals consider the diagnostic accuracy and reliability of the test to be the most important. Health professionals pointed out that the tests might be frequently performed without the pregnant women having adequate knowledge or counseling. © 2012 John Wiley & Sons, Ltd.

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Conflicts of interest: None declared.

INTRODUCTION

Despite recent human genetic breakthroughs and their applications in clinical practice, the prevention of genetic conditions in newborn still relies on screening, prenatal diagnosis, and termination of affected pregnancies. The decision to consent to prenatal diagnostic tests may be affected by a variety of factors, such as the country's culture, its health system, its abortion laws, and the information provided by the test, as well as parental level of awareness and education.^{1–4} It has been recognized that a prenatal health professional's attitudes may influence a pregnant woman's decision to consent to a prenatal diagnosis.^{5–7}

Invasive procedures such as amniocentesis and chorionic villus sampling (CVS) have been clinically available but involve small and definite risks to the fetus and/or the mother. To reduce the risk, a combination of maternal serum parameters and ultrasonographic screening is currently used to assess the risk of fetal aneuploidy. The development of new, potentially non-invasive approaches involves the examination of fetal cells or cell-free DNA within the maternal circulation as a source of

fetal DNA. The enrichment of nucleated red blood cells using lectin and a subsequent auto-detection system has improved the accuracy of the diagnosis of fetal aneuploidy^{8,9} and is now under evaluation in clinical trials. Another approach utilizes cell-free (extracellular) fetal DNA or RNA. The use of such procedures for determining the Rhesus factor status in RhD-negative pregnant women has been translated into clinical practice.¹⁰ In 2008, a next-generation sequencing technology was applied to cell-free DNA analysis in maternal plasma.^{11,12} The next-generation sequencing test was proven to be highly accurate for detecting fetal trisomy-21 in plasma,¹³ and it has been commercially approved for the non-invasive prenatal diagnosis (NIPD) of trisomy-21 in both the USA and China.

In Japan, prenatal genetic screening is performed in fewer than 5% of all pregnancies and mostly performed as second-trimester maternal serum screening. First-trimester screening is still uncommon in Japan. After maternal serum screening was introduced in the 1990s, the Ministry of Health, Labour, and Welfare of Japan published clinical practice guidelines

entitled "Opinions Concerning Tests using Maternal Serum Markers" in 1999. The guidelines concluded that the implementation of second-trimester maternal serum screening is not a necessity. Additionally, obstetricians were not obliged to actively inform pregnant women about second-trimester maternal serum screening. The guidelines also concluded that not all pregnant women should be informed about the availability of such testing. Nevertheless, when a pregnant woman requests an explanation about the test, the medical professional should carefully explain about the prenatal diagnosis. The consensus about the ultrasonography detection of soft markers, such as nuchal translucency, is followed by the Japan Society of Obstetrics and Gynecology because there were concerns about the ethical, social, and psychological aspects of the tests. For example, the test results might lead pregnant women to misunderstand the information or may make them anxious about the results expressed as part of an individual risk assessment.¹⁴ Therefore, there is concern about whether the test should be performed as mass screening to detect fetal abnormalities. For these reasons, Japanese health professionals tend not to actively inform pregnant women about prenatal screening tests (e.g., serum and ultrasonography) and diagnostic tests (e.g., amniocentesis and chorionic villus sampling). However, as pregnant women have learned more about genetic screening and prenatal diagnosis, there has recently been a greater demand for such testing.

New NIPD testing will be better in safety, robustness, and early time performed in pregnancy than invasive test. In the clinical setting, there is concern that informing parents about the prenatal screening can cause acceptance of this test and its results without sufficient consideration, causing an increase in elective abortion.^{7,15–17} Little is known about the attitudes of pregnant women regarding NIPD.^{18–21} Although Japan still has various limitations regarding the performance of both prenatal diagnosis and screening, it is important to know the opinions and social consciousness of pregnant women and health professionals to further develop NIPD testing systems in Japan.

METHODS

Self-report questionnaires were sent to clinical geneticists (pediatricians and obstetricians), midwives or nurses, and genetic counselors belonging to the same hospitals. Questionnaires were given to pregnant women when they visited the outpatient clinic at Showa University Hospital, Tokyo, and Shonan-Tobu Hospital, Kanagawa, Japan, between July and August 2011. A cover letter accompanied each questionnaire, explaining the purpose of the study, its confidentiality, and the method to follow when replying. Furthermore, in the supplement, we explain NIPD testing, which will become available as a first-trimester diagnostic test, as well as its risks, advantages, and disadvantages. The medical ethical committee of Showa University approved this study. Prior to the study, ten health professionals tested the questionnaire (the medical ethical committee of Showa University also approved this preliminary study). On the basis of the pilot test, the questionnaire required only minor grammatical changes.

The questionnaire was composed of seven sections. The first section collected sociodemographic data about the respondents and contained the information about the NIPD. The other six sections consisted questions relating to the following: (i) agreement or disagreement with the clinical application of NIPD; (ii) expectations about NIPD testing; (iii) concerns about the clinical application of NIPD; (iv) NIPD testing methods; (v) attitudes toward the clinical application of NIPD if the pregnant women have adequate or inadequate knowledge; and (vi) suitable targets of the NIPD testing. The survey used a 5-point Likert scale as follows: 1, fully disagree; 2, disagree; 3, neither agree nor disagree; 4, agree; 5, fully agree. Then, the 5-point Likert scale was limited to three categories (fully disagree, neither agree nor disagree, and fully agree) to avoid small data pools for some responses. A pregnant woman was assumed to have adequate knowledge when she knew the risks, advantages, and disadvantages of NIPD, as described in the questionnaire supplement. The data were analyzed using the Statistical Package for Social Sciences software program version 17. Cronbach's coefficient alpha test was used to clarify the validity of the test. The Chi-square test, Mann-Whitney test, Kruskal-Wallis test, and Friedman test were used in the statistical testing of differences between the groups.

RESULTS

Background characteristics

There were a total of 252 respondents consisting of 185 health professionals and 67 pregnant women. The response rate was 58% among obstetricians ($n=58$), 39% among pediatricians ($n=39$), and 45% among genetic counselors ($n=41$). The response rate of the pregnant women is unknown because the self-reported questionnaire was used at outpatient clinics. There were also 36 midwives or nurses in health professionals group. The demographic characteristics of the respondents are shown in Table 1.

Agreement or disagreement with the clinical application of NIPD

Table 2 shows that most of the health professionals and pregnant women agreed with the clinical application of NIPD. More pregnant women agree with the clinical application of NIPD than health professionals ($X^2=7.355$, $p<0.01$).

Expectations for NIPD

Table 3 shows the answers to the 11 questions related to the expectation for NIPD as follows: the better safety of the NIPD, the lower maternal physical and psychological stress related to the test's procedure, the earlier gestational age of the testing performed, the higher diagnostic accuracy, the ability to provide a definitive diagnosis, and the shorter processing time for the results (on the 5-point Likert scale). Pregnant women strongly expected the testing to be safe for the fetus and to result in less maternal physical and psychological stress. The medical professionals strongly expected the tests to be safe for the fetus, to have high diagnostic accuracy, and to provide a definitive diagnosis. It was revealed that the

Table 1 The demographic characteristics of the respondents

		Health professionals (N= 185)	Pregnant women (N= 67)
Gender	Female	98 (53%)	67 (100%)
	Male	81 (44%)	
	Not stated	6 (3%)	
Age	20–29	16 (8.6%)	10 (14.9%)
	30–39	34 (18.4%)	44 (65.7%)
	40–49	65 (35.1%)	10 (14.9%)
	50–59	57 (30.8%)	
	>60	10 (5.4%)	
	Not stated	3 (1.7%)	3 (4.5%)
Occupation	Obstetrician/clinical geneticist	58 (31.3%)	
	Pediatrician/clinical geneticist	39 (21.1%)	
	Genetic counselor	41 (22.2%)	
	Midwife or nurse	36 (19.5%)	
	Other	11 (5.9%)	

Table 2 Agreement or disagreement with the clinical application of non-invasive prenatal diagnosis testing

	Medical professionals	Pregnant women	Total
Agree	97 (78.9%)	38 (97.3%)	135 (83.3%)
Disagree	26 (21.1%)	1 (2.7%)	27 (16.7%)
Total	123	39	162

pregnant women expected more from the NIPD than the medical professionals in seven of the 11 items (Mann–Whitney test), including less maternal physical and psychological stress, items associated with an earlier diagnosis, and those associated with the safety of the NIPD tests.

Table 3 Expectations about non-invasive prenatal diagnosis testing

	Averaged rank ^a		p-value ^b
	Medical professionals	Pregnant women	
Safe for the fetus	8.47	7.83	0.045
Less maternal physical and physiological stress (because of peripheral blood sampling)	6.86	7.15	0.000
Can be diagnosed early in pregnancy (~10 weeks)	6.38	5.10	0.233
High diagnostic accuracy (compared with amniocentesis)	7.22	6.56	0.144
Definitive diagnosis (instead of screening)	7.20	6.69	0.065
Shorter processing time for results (about 1 week)	6.75	5.52	0.277
Earlier results are better because they reduce concerns about the baby	4.33	6.71	0.000
Earlier results lead to a better relationship between the mother and the newborn	3.27	4.40	0.000
Earlier treatment/consideration can be performed if an abnormality is found	5.03	5.95	0.000
In case of abortion, there is less maternal stress because of earlier gestation	4.66	4.36	0.003
Results are easy to understand	5.83	5.73	0.001

^aFriedman test: In each group of medical professionals and pregnant women, a rank sum analysis was performed. The Friedman test [non-parametric statistical test] is used to detect differences in paired multiple items and is indicated by the rank sum result.

^bMann–Whitney test: The difference between medical professionals and pregnant women was analyzed.

Concerns about the clinical application of NIPD

Table 4 shows the responses to the 16 questions related to the concerns about the clinical applications of NIPD (on a 5-points Likert scale). Medical professionals had numerous concerns, especially with regard to the test's implementation if the pregnant women have inadequate knowledge of the NIPD, insufficient counseling before the testing, the occurrence of unexpected results and confusion, and increased test participation without full consideration. On the other hand, the pregnant women had concerns about an unequal access to the test (because of the cost), test's implementation without NIPD knowledge, an increase in abortions, and an increase in test participation without full consideration. Health professionals are significantly more concerned about NIPD than the pregnant women in ten of the 15 items ($p < 0.001$) as follows: increased test participation

Table 4 Concerns about the clinical application of NIPD testing

	Averaged rank ^a		p-value ^b
	Medical professionals	Pregnant women	
Increase in test participation without full consideration	10.41	9.56	0.000
Autonomous option of the test is restricted	6.88	6.17	0.000
Unequal access to the test due to cost	6.96	10.82	0.052
More pregnant women would receive an unexpected result (because of the frequency of testing), causing confusion	11.25	10.07	0.000
Counseling is insufficient because of the non-invasiveness of the testing	11.30	7.59	0.000
Abortions would become more prevalent	7.61	10.38	0.947
Autonomous decisions to have a baby with a chromosomal abnormality will be limited	6.47	9.36	0.992
Testing would be performed more often with inadequate physician's knowledge	10.67	7.24	0.000
Testing would be performed with inadequate pregnant women's knowledge	11.40	10.59	0.000
NIPD cannot diagnose chromosomal structural abnormalities (e.g., translocation)	9.01	7.63	0.000
NIPD can lead to discrimination against newborns with chromosomal abnormalities because of decreased prevalence	5.51	7.51	0.427
Unnecessary testing would be performed	8.82	7.78	0.000
Testing will develop into broad and global genetic screening	8.22	7.41	0.000
Testing will develop into mass screening	6.95	8.02	0.082
Testing will develop for non-medical purposes (e.g., gender determination)	7.08	6.93	0.000
The technology may be diverted for the sake of developing a "designer baby"	7.48	8.97	0.07

NIPD, non-invasive prenatal diagnosis.

^aFriedman test: In each group of medical professionals and pregnant women, the rank sum analysis was performed.

^bMann-Whitney test: The difference between medical professionals and pregnant women was analyzed.

without full consideration, inadequate counseling prior to the test, implementation of such tests without NIPD knowledge among medical professionals and pregnant women, and using such tests when not necessary.

Clinical application of NIPD testing

In this section, we asked medical professionals and pregnant women whether each survey participant accepts the clinical setting that all pregnant women be allowed to undergo NIPD testing if they want to have the test. The attitudes of the pregnant women and the healthcare professionals differed in regard to this question (Table 5). Among the pregnant women, 97.8% agreed that women should be allowed to undergo the test if they want it, whereas only 35.9% of the healthcare professionals agreed ($X^2 = 49.049, p < 0.001$).

We also asked whether NIPD testing should be limited to women with a significant risk for having a fetus with a genetic condition (Table 6). Both groups showed a similar tendency:

Table 5 Pregnant women are able to undergo non-invasive prenatal diagnosis testing if they so desire: medical professionals versus pregnant women

	Medical professionals	Pregnant women	Total
Agree	42 (35.9%)	43 (97.8%)	85 (52.8%)
Disagree	75 (64.1%)	1 (2.2%)	76 (47.2%)
Total	117	44	161

namely, a total of 84.8% of the health professionals and 77.8% of the pregnant women agreed that this test should be performed in high-risk cases ($p = 0.217$).

Attitudes about the clinical application of NIPD with or without sufficient knowledge about the test

Table 7 shows that respondents with inadequate NIPD knowledge tended to have a positive attitude toward the clinical application of NIPD (76.8%). Only 34.3% of respondents with adequate NIPD knowledge agreed that pregnant women be allowed to undergo NIPD test if they want to ($X^2 = 22.569, p < 0.0001$). Table 8 shows that 36.6% and 60.5% respondents, with inadequate or adequate NIPD knowledge, respectively, wanted their partner to undergo the test ($X^2 = 5.717, p < 0.05$).

Suitable targets for NIPD testing

In order of importance, the suitable targets for NIPD testing according to health professionals are as follows: childhood-onset

Table 6 Non-invasive prenatal diagnosis testing should be performed for pregnant women who have a significant risk for having a fetus with a genetic condition

	Medical professionals (N=185)	Pregnant women (N=67)	Total
Agree	123 (84.8%)	28 (77.8%)	30 (16.6%)
Disagree	22 (15.2%)	8 (22.2%)	151 (83.4%)
Total	145	36	181

Table 7 Acceptance of the clinical application of NIPD testing with and without knowledge about the NIPD

	With knowledge about NIPD	Without knowledge about NIPD	Total
Agree	24 (34.3%)	43 (76.8%)	67 (53.2%)
Disagree	46 (65.7%)	13 (23.2%)	59 (46.8%)
Total	70	56	126

NIPD, non-invasive prenatal diagnosis.

Table 8 Respondents hope to undergo the NIPD testing if they or their partner becomes pregnant

	With knowledge about NIPD	Without knowledge about NIPD	Total
Agree	26 (36.6%)	23 (60.5%)	49 (45%)
Disagree	45 (63.4%)	15 (39.5%)	60 (55%)
Total	71	38	109

NIPD, non-invasive prenatal diagnosis.

Table 9 Suitable targets for non-invasive prenatal diagnosis testing based on the opinion of medical professionals

	Averaged rank ^a
Childhood-onset severe genetic diseases	11.13
At risk of severe X-linked diseases	10.91
RhD blood typing	10.64
Chromosomal aneuploidy (e.g., Down syndrome)	10.63
Chromosomal abnormality (e.g., microdeletion, duplication)	8.82
Chromosomal test equal to karyotyping quality	8.32
Adult-onset severe neurological disorder (e.g., Huntington's disease)	8.12
Adult-onset hereditary cancer	7.28
Carrier testing for genetic disorders	5.91
Mild phenotype genetic disorders (e.g., color blindness)	5.64
Broad and global identification of genetic conditions	5.24
Lifestyle-related diseases (e.g., diabetes)	4.77
Mass-screening applications	4.54
Gender identification without medical reason	3.03

^aFriedman test.

severe genetic disorders, severe X-linked disorders, rhesus D blood typing, and chromosomal aneuploidy such as Down syndrome and trisomy-18 (Table 9). The healthcare professionals disagreed about the application of NIPD testing for gender determination without a medical reason and for adult-onset diseases as mass screening for all pregnant women.

DISCUSSION

The present study showed that most of the pregnant women and health professionals had positive attitudes toward the clinical application of NIPD, although around 20%, especially clinical geneticists, were reluctant to recommend NIPD usage in clinical practice. These positive attitudes could hasten the acceptance of the application of NIPD testing in clinical

practice, especially in Japan. There were discrepancies in the expectations and indications for NIPD between healthcare professionals and pregnant women. In particular, the pregnant women expected most in fetal safety and less in maternal stress of NIPD. Meanwhile, medical professionals expected most in high diagnostic accuracy, the ability to provide a definitive diagnosis, and the safety of the testing. Furthermore, pregnant women considered the reduction of maternal stress during the test procedure and the ability to have an earlier diagnosis (to relieve anxiety) to be more important than did the health professionals. Medical professionals put more importance on the diagnostic accuracy and reliability of the test. These differences in expectations and concerns could help in the development of improved education strategies for both pregnant women and health professionals.

Regarding concerns about the clinical application of NIPD, healthcare professionals pointed out that the test may be performed without providing information about the advantages and limitations of NIPD because of the test's non-invasiveness. In this study, the pregnant women are concerned about unequal access to the test due to the cost and increase in abortions performed.

It is interesting to note that those respondents with inadequate knowledge of NIPD tended to appreciate NIPD testing. The cause of this tendency is unknown. There was a possibility that pregnant women might accept NIPD testing more easily because of an unquestioned acceptance of anything new.¹⁸ Moreover, in this study, more pregnant women than health professionals agreed that pregnant women be allowed to undergo the test if they wish. In this section, when we presented the first statement, "All women are able to undergo NIPD testing if they want it," there was a discrepancy in opinions (97.8% pregnant women agreed, 35.9% health professionals agreed, $p < 0.001$). Then, in the second statement, "NIPD should be limited to women with a high risk of a genetic condition," there was no substantial discrepancy in opinions (77.8% pregnant women agreed, 84.8% health professionals agreed, $p = 0.217$). When presented with the second statement, the pregnant women might have reconsidered, and their attitudes were changed slightly. This also indicates that pregnant women might accept NIPD testing more easily because of an unquestioned acceptance.

This difference in tendencies could lead to several problems. If the test is conducted on pregnant women with inadequate knowledge about the purpose of the test and what kinds of information can be obtained, the test results might thus create either misunderstanding or anxiety.

Although the survey did not address these problems, providing sufficient information about NIPD to both pregnant women and health professionals might have a positive impact. Prior to NIPD testing, it is necessary that genetic counseling, including discussions about the benefits and limitations of testing, is provided to every pregnant woman. In this way, pregnant women could make an autonomous informed decision about using NIPD and the test's results.²²

The NIPD technology became commercially available in the USA in 2011, but only the test for trisomy-21 is available as an advanced screening test with an extremely high detection rate and a low false-positive rate. As a result, the International Society

for Prenatal Diagnosis has announced a statement about "Prenatal Detection of Down Syndrome Using Massively Parallel Sequencing (MPS)" (<http://cmg.informz.net/CMG-ISP/~/media/ispd.rr.mps.24oct11.pdf>). This statement indicates that MPS can be helpful for pregnant women who may have been determined to be at high risk by previously recommended screening methods under suitable genetic counseling and that the International Society for Prenatal Diagnosis does not endorse the *ad hoc* use of MPS testing in women at lower risk. However, the future directions of NIPD technology will likely hasten progress toward definitive testing because pregnant women are hoping for the establishment of safe and reliable tests. Therefore, at the time of this MPS clinical application, a counseling system should be implemented for pregnant women prior to and after testing. Pregnant women who are considering the MPS test need to receive detailed genetic counseling and to give their informed consent prior to the test. The present study suggests that genetic counseling prior to and after the testing is vital to addressing the challenges associated with building a new framework for genetic counseling for NIPD testing.

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WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- New non-invasive prenatal diagnosis testing will be better than invasive test in safety, definitive result, robustness, and early time period in pregnancy. There were concerns about the clinical application of the non-invasive prenatal diagnosis; however, the details of the concerns among both pregnant women and health professionals have not yet been assessed in Japan.

WHAT DOES THIS STUDY ADD?

- We revealed the attitudes toward non-invasive prenatal diagnosis (NIPD) among pregnant women and health professionals. There were discrepancies in concerns and expectations about NIPD, between the health professionals and the pregnant women. It is suggested that genetic counseling prior to and after the testing is vital to addressing the challenges in building a new framework for genetic counseling for NIPD testing.

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Brain magnetic resonance imaging and motor and intellectual functioning in 86 patients born at term with spastic diplegia

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ABBREVIATIONS

PVI Periventricular venous infarction
PVL Periventricular leukomalacia

AIM To investigate the association between magnetic resonance imaging (MRI) patterns and motor function, epileptic episodes, and IQ or developmental quotient in patients born at term with spastic diplegia.

METHOD Eighty-six patients born at term with cerebral palsy (CP) and spastic diplegia (54 males, 32 females; median age 20y, range 7–42y) among 829 patients with CP underwent brain MRI between 1990 and 2008. The MRI and clinical findings were analysed retrospectively. Intellectual disability was classified according to the Enjoji developmental test or the Wechsler Intelligence Scale for Children (3rd edition).

RESULTS The median ages at diagnosis of CP, assignment of Gross Motor Function Classification System (GMFCS) level, cognitive assessment, and MRI were 2 years (range 5mo–8y), 6 years (2y 8mo–19y), 6 years (1y 4mo–19y), and 7 years (10mo–30y) respectively. MRI included normal findings (41.9%), periventricular leukomalacia, hypomyelination, and porencephaly/periventricular venous infarction. The frequency of patients in GMFCS levels III to V and intellectual disability did not differ between those with normal and abnormal MRI findings. Patients with normal MRI findings had significantly fewer epileptic episodes than those with abnormal ones ($p=0.001$).

INTERPRETATION Varied MRI findings, as well as the presence of severe motor dysfunction and intellectual disability (despite normal MRI), suggest that patients born at term with spastic diplegia had heterogeneous and unidentified pathophysiology.

Spastic diplegia is a subgroup of cerebral palsy (CP) found both in preterm and term infants; however, the underlying causes differ. Preterm spastic diplegia is the result of periventricular leukomalacia (PVL),¹ whereas only 12% of children born at term with spastic diplegia have PVL.² In a study based on the data collected from the Surveillance of Cerebral Palsy in Europe collaboration, 45.7% of patients born at term with CP with a birthweight of 2500g or more had bilateral spastic palsy.³ The ratio of patients with CP with severe impairments peaked in the 1980s, fell during 1990 to 1999, and was little changed in the 1990s compared with the late 1970s.⁴ However, another recent population-based comparative analysis of children with CP born between 1990 and 1996, and between 1997 and 2003 showed an increase in those born at term with spastic diplegia from 9 (4/46) to 20% (5/25)⁵ respectively. The pathogenesis of spastic diplegia in those born at term is poorly understood. Although some clinical and neuroradiological studies have reported magnetic resonance imaging (MRI) findings other than PVL,^{1,6–9} most used small sample

sizes and, thus, did not provide a broad perspective on the pathology of spastic diplegia in those born at term.

The present study investigated the relation between MRI and clinical findings in a large sample of children born at term with spastic diplegia.

METHOD

Patients

Between 1 September 1990 and 31 August 2008, 829 patients with CP underwent MRI at the Takuto Rehabilitation Center for Children, Sendai, Japan. Of those, we enrolled 86 term patients (54 males, 32 females; median age 20y, range 7–42y) with clinical evidence of spastic diplegia. The inclusion criteria were a diagnosis of CP, a diagnosis of spastic diplegia, and gestational age of 37 weeks or more. Spastic diplegia was defined as a type of spastic quadriplegia affecting the lower more than the upper extremities.^{1,6,7} Brain MRI and clinical findings of the participants were retrospectively analysed. The present study was approved by the ethics

committee of the Takuto Rehabilitation Center for Children (number 22-2).

MRI analysis

MRI was performed using a Toshiba MRIT 50A scanner (0.5T; Toshiba, Tokyo, Japan) with 10mm multislice axial and sagittal T1-weighted spin-echo images (repetition time/echo time [TR/TE] 375/14) and axial T2-weighted spin-echo images (TR/TE 2000/120), or a Shimadzu-Marconi Magnex Eclipse scanner (1.5T; Shimadzu-Marconi, Kyoto, Japan) with 5mm multislice axial and sagittal T1-weighted images (TR/TE 500/20) and axial T2-weighted images (TR/TE 5000/72). Two authors (YN, IS-S) with no knowledge of clinical information independently evaluated the MRI findings. The MRI findings were categorized as shown in Table I and are illustrated in Figure 1. Hypomyelination was defined as a delayed or arrested normal myelinating process resulting in a persistent immature pattern of myelination in the cerebral white matter. Porencephaly was defined as fluid-filled cavities in the cerebral hemisphere connecting to the lateral ventricle. In the present study, there were no patients with porencephaly connected to the hemisphere surface. Periventricular venous infarction (PVI) was defined to the loss of periventricular tissues after venous infarction caused by subependymal haemorrhage.¹⁰ Because porencephaly and PVI are probably based on the same pathophysiology, we combined them into the one category. Cerebellar atrophy was defined as a small cerebellum with prominent fissures between shrunken folia,¹¹ whereas cerebellar hypoplasia was defined as a small cerebellum with normally sized fissures and folia.¹¹ When MRI revealed mixed abnormalities, the diagnosis was based on the most prevalent and dominant findings as the cause of the spastic diplegia. Furthermore, patients with abnormalities in more than one radiological category were analysed. Disagreements in diagnosis were resolved by the third author (YK) and second author (AO).

What this paper adds

- Almost 42% of patients born at term with spastic diplegia had normal MRI findings.
- The frequency of patients with severe motor dysfunction and intellectual disability did not differ between those with normal and abnormal MRI findings.

Clinical assessment

The patients' age at diagnosis of CP, assignment of Gross Motor Function Classification System (GMFCS)¹² level, and cognitive assessment as well as the history of asphyxia were retrospectively analysed from the medical records.

Motor-deficit severity was classified into levels I to V according to the GMFCS; the levels were interpreted from the latest clinical records. We found it difficult to distinguish between GMFCS levels I and II clinically; thus, those levels were combined in the statistical analysis.

The children's developmental quotients (DQ) or intelligence quotients (IQ) were assessed using either the Enjoji developmental test, which evaluates physical abilities of the whole body, skilled hand motor activities, behaviour, interpersonal skills, speech ability, and language comprehension,¹³ or the Wechsler Intelligence Scale for Children (3rd edition).¹⁴ In cases of serial rating studies, we used the latest result to ensure an exact classification. The patients' DQ/IQs were graded and classified into three groups: normal, DQ/IQ \geq 70; mild, 50 \leq DQ/IQ $<$ 70; severe, DQ/IQ $<$ 50.

Epilepsy was defined as the occurrence of at least two unprovoked epileptic seizures; neonatal seizures and febrile convulsions were not included.¹⁵ The epilepsy outcome at the time of the present study was classified as good when the patient was seizure-free for more than 2 years, poor when the patient had more than two seizures per month despite appropriate treatment, and moderate if the patient was between the two classifications.

Table I: Magnetic resonance imaging classification and its distribution among patients born at term with spastic diplegia

Classification	Description	n	%
Normal	No abnormality detected	36	41.9
Periventricular leukomalacia	Symmetric reduction of the peri-trigonal white matter volume with a consecutive enlargement of the posterior horns, scalloped ventricular contours, periventricular gliosis and secondary atrophy of the posterior part of the body and the splenium of the corpus callosum	12	14.0
Malformation	Includes cortical dysplasia, schizencephaly, polymicrogyria, pachygyria, and heterotopias	3	3.5
Hypomyelination	Delayed or arrested normal myelinating process resulting in persistent immature pattern of myelination in the cerebral white matter	5	5.8
Cerebellar atrophy	Shrinkage of cerebellar vermis and hemisphere	2	2.3
Diffuse cortical atrophy	Diffuse reduction of the volume of cerebral gyri	4	4.7
Enlargement of the bilateral lateral ventricles	Enlargement of bilateral lateral ventricles except the findings of periventricular leukomalacia	12	14.0
Border-zone infarction	Ischemic changes in the watershed area between anterior and medial cerebral artery or between medial and posterior cerebral artery	3	3.5
Porencephaly/periventricular venous infarction	Fluid-filled cavities in the cerebral hemisphere connecting to the lateral ventricle/loss of periventricular tissues after venous infarction caused by subependymal haemorrhage	6	7.0
Thin corpus callosum	Thinning of the corpus callosum except the findings of periventricular leukomalacia and porencephaly	1	1.1
Unclassifiable	Unable to be classified into one of the above groups	2	2.3
Total		86	

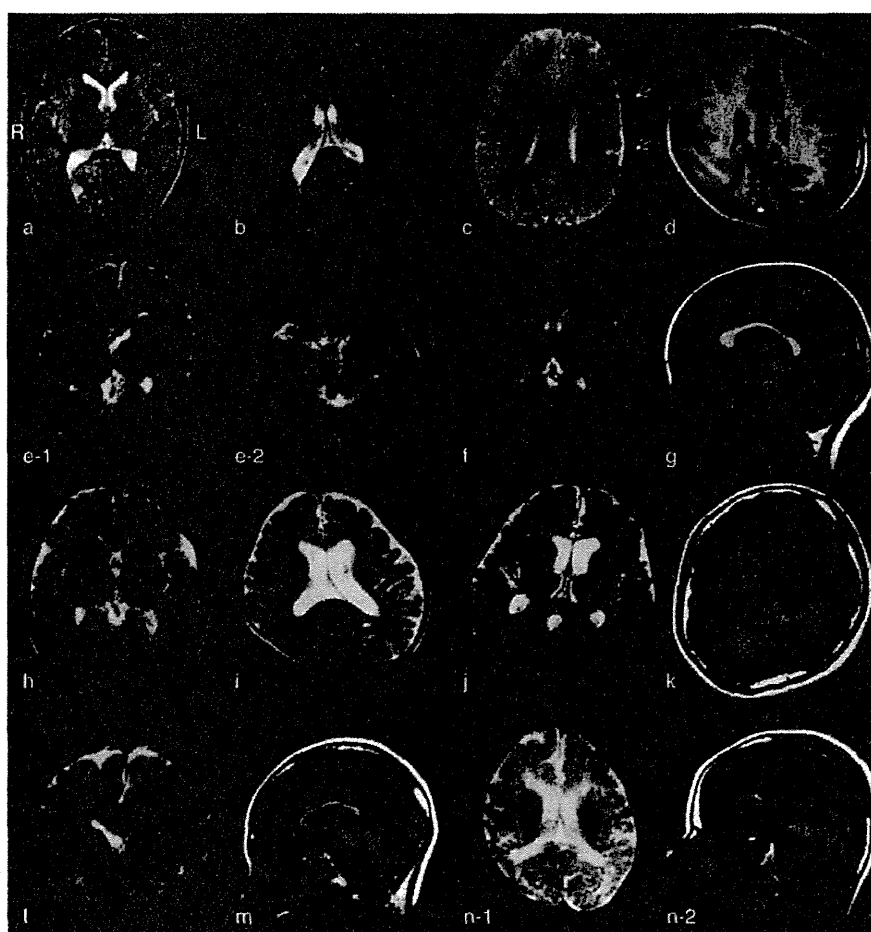


Figure 1: Magnetic resonance imaging findings of term patients with spastic diplegia. (a) Normal findings detected by T2-weighted spin-echo images (T2WI) at 7 years. (b) Periventricular leukomalacia by T2WI at 6 years. (c) Hemispheric polymicrogyria of the left hemisphere (arrows) by T2WI at 4 years. (d) Bilateral parieto-occipital schizencephaly by T1-weighted spin-echo images (T1WI) at 5 years. (e-1, e-2) Cerebral and cerebellar malformation by T1WI at 3 years. (f) Hypomyelination by T2WI performed at 10 years. (g) Cerebellar atrophy by T1WI at 10 years. (h) Diffuse cortical atrophy by T2WI at 10 years. (i) Enlargement of the bilateral lateral ventricles by T2WI at 2 years. (j) Bilateral frontal border-zone infarction by T1WI at 3 years. (k) Porencephaly by T1WI at 30 years. (l) Periventricular venous infarction by T2WI at 15 years. (m) Thin corpus callosum by T1WI at 12 years. (n-1, n-2) MRI images of the patients with hypomyelination, enlargement of the bilateral lateral ventricles, diffuse cortical atrophy, and thin corpus callosum at 7 years.

Table II: Correlation between normal or abnormal magnetic resonance imaging findings and Gross Motor Function Classification System (GMFCS) level, epilepsy, and intellectual disability

	All (n=86)		Normal (n=36)		Abnormal (n=50)		p
	n	%	n	%	n	%	
GMFCS level							
I or II	61	70.9	30	83	31	62	0.053
III-V	25	29.1	6	7	19	38	
Incidence of epilepsy	24	27.9	3	8	21	42	0.001
Outcome of epilepsy							
Good	14		3		11		
Moderate	4		0		4		
Poor	6		0		6		
Intellectual disability	52	60.5	19	51	33	66	0.266
Mild	15		7		8		
Severe	37		12		25		

Intellectual disability levels: normal, $DQ/IQ \geq 70$; mild, $DQ \leq 50/IQ < 70$; severe, $IQ/DQ < 50$.

Relationships between normal or abnormal MRI findings and GMFCS level, epilepsy, and intellectual disability are shown in Table II.

Statistical analysis

The association between factors in the children's clinical profile (motor function, epilepsy, and intellectual disability) and MRI findings was investigated. Statistical analyses were conducted using a χ^2 test. A p value <0.05 was deemed statistically significant.

RESULTS

The median ages at diagnosis of CP, assignment of GMFCS level, cognitive assessment, and MRI were 2 years (range 5mo–8y), 6 years (2y 8mo–19y), 6 years (1y 4mo–19y), and 7 years (10mo–30y) respectively. Seventeen patients experienced birth asphyxia, including those with normal MRI ($n=4$), PVL ($n=5$), diffuse cortical atrophy ($n=2$), hypomyelination ($n=2$), porencephaly/PVI ($n=1$), enlargement of the bilateral lateral ventricles ($n=1$), thin corpus callosum ($n=1$), and border-zone infarction ($n=1$).

MRI findings

The MRI results of the 86 patients born at term with spastic diplegia (Table I) revealed normal findings in 36 (41.9%) patients (Fig. 1a) and abnormal findings in 50 (58.1%). We examined 60 patients and 26 patients by 0.5T and 1.5T MRI respectively. The proportion of normal findings did not significantly differ between 0.5T and 1.5T MRI ($p=1.0$).

Of the abnormal MRI findings, PVL was observed in 12 patients (Fig. 1b), malformation was found in three, hemispheric polymicrogyria of the left hemisphere in one (Fig. 1c), bilateral parieto-occipital schizencephaly in one (Fig. 1d), and cerebral and cerebellar malformation in one (Fig. 1e-1, e-2). Hypomyelination was observed in five (Fig. 1f), cerebellar atrophy in two (Fig. 1g), diffuse cortical atrophy in four (Fig. 1h), enlargement of the bilateral lateral ventricles in 12 (Fig. 1i), border-zone infarction in three (Fig. 1j), porencephaly (Fig. 1k)/PVI (Fig. 1l) in six, and thin corpus callosum in one (Fig. 1m). Two patients were unclassified according to the above system.

Motor impairment

The associations between MRI findings and motor-deficit severity, epilepsy, and cognitive impairment are shown in Table II. Of the 86 patients, 61 (69%) were classified in GMFCS levels I or II. Of the 36 patients with normal MRI, 30 (83%) were graded in GMFCS levels I or II, whereas 30 of 50 patients (62%) with abnormal MRI findings were independently ambulatory (GMFCS levels I or II). These results indicate the frequency of patients with motor dysfunction did not differ between those with normal and abnormal MRI findings ($p=0.053$). The present study identified 25 patients in GMFCS levels III to V. Their MRI findings included normal findings ($n=6$), PVL ($n=3$), malformation ($n=2$), hypomyelination ($n=3$), cerebellar atrophy ($n=2$), diffuse cortical atrophy ($n=2$), enlargement of the bilateral lateral ventricles ($n=2$),

border-zone infarction ($n=1$), porencephaly/PVI ($n=2$), thin corpus callosum ($n=1$), and unclassified ($n=1$). They contained five patients with abnormalities in more than one radiological category: one with cerebellar hypoplasia and pachygyria plus thin corpus callosum and enlargement of bilateral lateral ventricles; one with diffuse cortical atrophy plus enlargement of bilateral lateral ventricles; two with hypomyelination plus thin corpus callosum; and one with hypomyelination plus thin corpus callosum plus enlargement of bilateral lateral ventricles plus diffuse cortical atrophy (Fig. 1n-1, n-2). The first category was evaluated with the most dominant findings adopted as the MRI classification in Table II.

Epilepsy

Of the 86 patients, 24 (27.9%) had a history of epilepsy, including three of the 36 patients (8%) with normal MRI and 21 of the 50 patients (42%) with abnormal MRI findings. Thus, the frequency of epilepsy was significantly lower in patients with normal MRI findings than in those with abnormal ones ($p=0.001$). Among the 24 patients with epilepsy, 14 (58%) exhibited good control, four (17%) had moderate control, and six (25%) exhibited poor control. The MRI findings of the six patients with poorly controlled epilepsy were as follows: porencephaly/PVI ($n=2$), PVL ($n=1$), malformation ($n=1$), hypomyelination ($n=1$), and enlargement of the bilateral lateral ventricles ($n=1$). MRI of one patient who developed West syndrome revealed that hypomyelination overlapped diffuse cortical atrophy and enlargement of the bilateral lateral ventricles; the patient had severe intellectual disability, a poor epilepsy outcome, and was classified in GMFCS level V.

Intellectual disability

Of the 86 patients, 52 (60.5%) had an intellectual disability. In the group of 36 patients with normal MRI findings, 19 (51%) exhibited intellectual disability, including 12 (63%) with severe disability and seven (37%) with mild disability. Similarly, 33 of the 50 (66%) patients with abnormal MRI findings had intellectual disability; 25 (76%) severely and eight (24%) mildly. These results indicate that the MRI findings were not correlated with the frequency of intellectual disability in patients born at term with spastic diplegia ($p=0.266$).

Other characteristic findings

Two patients showed both cerebellar hemispheric and vermi-an atrophy. One had no history of asphyxia, the other had no perinatal record. They were referred to our hospital because of delayed motor development and crouched posture, and were diagnosed with spastic diplegia at the ages of 2 and 9 years 7 months respectively. Both had tendon-lengthening surgery around the age of 10 years. One had intellectual disability and epilepsy. One was non-ambulatory, whereas the other one was ambulatory with crutches.

Five patients (three males, two females) whose diagnosis of spastic diplegia was made between 2 and 3 years of age had hypomyelination. Their final MRI was studied at 3, 7, 8, 10, and 11 years respectively. MRI results showed severe hypomyelination with reduced white matter volume, whereas the

thalamus, brainstem, and cerebellum showed preserved myelination. Three patients had a history of birth asphyxia. Two of these showed ventricular dilatation and cortical atrophy. Four patients had severe intellectual disability. Three had epilepsy. Three patients were non-ambulatory, whereas two were able to walk independently. Auditory brainstem response, which correspond to the electrophysiological activity of the auditory system throughout the brainstem,¹⁶ was abnormal in three patients in whom the upper brainstem components were not detected; two of these patients were siblings (one male, one female), suggesting an autosomal recessive trait. The *PLP1*, *GJC2*, and myelin basic protein gene analyses were normal in one patient. No patient showed clinical deterioration.

Eight patients had asymmetric MRI abnormalities. The MRI findings in these patients were porencephaly/PVI ($n=5$), malformation (hemispheric polymicrogyria; $n=1$), cerebellar atrophy ($n=1$), and enlargement of the bilateral lateral ventricles ($n=1$). Five patients with porencephaly/PVI and one patient with malformation had clinical asymmetry, which was dominant in the contralateral extremities.

Eight patients had abnormalities in more than one radiological category, as follows: enlargement of bilateral lateral ventricles plus thin corpus callosum ($n=1$) or diffuse cortical atrophy ($n=1$); malformation, which was cerebellar hypoplasia and pachygyria plus thin corpus callosum and enlargement of bilateral lateral ventricles ($n=1$); hypomyelination plus thin corpus callosum ($n=2$) plus enlargement of bilateral lateral ventricles ($n=1$) and diffuse cortical atrophy ($n=1$); and diffuse cortical atrophy plus enlargement of bilateral lateral ventricles ($n=1$). They included five patients in GMFCS levels III to V, four patients with epilepsy, and six patients with intellectual disability.

DISCUSSION

The results of the present study show that the MRI findings were more varied in those born at term with spastic diplegia than those born preterm with spastic diplegia, suggesting that the pathogenesis of spastic diplegia in those born at term is heterogeneous. A recent meta-analysis involving four studies of patients with spastic CP identified 51 term-born patients

with spastic diplegia.¹⁷ The MRI findings were classified as normal in 24 (47%), PVL in 13 (25.5%), brain maldevelopment in 6 (11.8%), grey matter lesions in four (7.8%), and miscellaneous in four (7.8%).¹⁷ In our study, 36 patients (41.9%) were found to have normal MRI findings, which is consistent with previous reports (Table III). Our study demonstrated that patients born at term with spastic diplegia who had normal MRI findings showed significantly fewer epileptic episodes than those with abnormal MRI findings. Patients born at term with spastic diplegia and normal MRI findings tended to show less severe motor dysfunction than those with abnormal MRI findings, although it was not significant. However, the percentage of patients with intellectual disability did not differ between the normal (51%) and the abnormal (66%) MRI groups.

Previous studies of patients with preterm spastic diplegia have found that the severity of motor disability was correlated with the degree of white matter reduction.⁶ However, an Australian population-based cohort study of CP by Robinson et al.¹⁸ reported that spastic diplegia was the most common clinical motor impairment in patients with normal MRI findings (12/25). These findings suggest the existence of unknown pathophysiological processes. In our study, six patients were classified in GMFCS levels III to V despite their normal MRI findings. Additionally, the proportion of cerebellar atrophy and hypomyelination was high in the patients in GMFCS levels III to V.

Individuals with CP and epilepsy have been reported to have structural brain abnormalities.¹⁹ Our results are consistent with these earlier reports. Previous studies have reported that 18 to 39% of patients with spastic diplegia develop epilepsy.¹⁹⁻²² Among them, a comparative analysis of the frequency of epilepsy in those born preterm and at term with spastic diplegia revealed that epilepsy was more common in those born at term.²² The predominance of deep white matter lesions in preterm infants is thought to be one of the factors explaining why preterm patients with spastic diplegia are less likely to develop epilepsy.^{22,23} We found that the frequency of epilepsy was significantly higher in patients with abnormal MRI findings than in those with normal ones.

Table III: Patients born at term with spastic diplegia who had normal magnetic resonance imaging (MRI) and characteristics of spastic diplegia

References	Number born at term with spastic diplegia	Number born at term with spastic diplegia and normal MRI findings (%)	Characteristics of spastic diplegia (n born at term/preterm) (%)			
			Spastic diplegia (term/preterm)	Spastic diplegia (term/preterm) with severe motor impairment	Spastic diplegia (term/preterm) with epilepsy	Spastic diplegia (term/preterm) with intellectual disability
Koeda et al. ¹	7	4 (57)	18 (7/11)	-	-	-
Yokochi et al. ⁶	3	2 (67)	34 (3/31)	20 (-/-) (58.8)	-	20 (-/-) (58.8)
Krägeloh-Mann et al. ⁹	9	2 (22)	29 (9/20)	-	-	-
Hayakawa et al. ⁷	20	11 (55)	63 (20/43)	6 (1/5) (9.5)	-	-
Okumura et al. ⁸	13	4 (31)	81 (13/68)	-	-	-
Carlsson et al. ²⁰	23	-	59 (23/36)	-	21 (12/9) (35.6)	-
Kulak et al. ²¹	17	-	40 (17/23)	5 (-/-) (12.5)	7 (-/-) (17.5)	30 (-/-) (75)
Present study	86	36 (41)	(86/-)	(25/-)	(24/-)	(52/-)

-, not examined.

Surprisingly, there was no difference in the frequency of intellectual disability between groups with normal and abnormal MRI findings. To the best of our knowledge, there are no reports on the relationship between severity of cognitive deficits and MRI findings in patients born at term with spastic diplegia. Reports comparing cognitive abnormalities in preterm and term-born children with spastic diplegia with PVL showed that visuoperceptual defects were common in preterm children.²⁴ Veelken et al.²² reported that one-quarter of their children born at term with diplegia had severe intellectual disability (IQ<50), in contrast to 5% among preterm children with diplegia. In our study, the proportion of patients with intellectual disability, 60.5% (52/86) of all patients born at term with spastic diplegia, might be relatively high. Over 50% of patients born at term with spastic diplegia with normal MRI had intellectual disability. 'Normal MRI findings' may reflect as-yet undetected abnormalities by widely used MRI scanners. Furthermore, the use of advanced brain imaging techniques with an examination of genetic or early prenatal factors²⁵ may be helpful in elucidating the nature of spastic diplegia in those born at term.

PVL and porencephaly/PVI are categorized as preterm-type brain injuries because they are the result of vascular system immaturity, where vascular watershed areas are situated in the periventricular white matter.²⁶ This type of injury typically

causes spastic diplegia based on the somatotopic organization of descending corticospinal tracts.²⁶ In the present study, PVL and porencephaly/PVI were observed in 18 patients with spastic diplegia; all but one, whose perinatal medical record was not available, showed no evidence of perinatal asphyxia, suggesting that PVL and porencephaly/PVI in the term infants occurred in utero early in the third trimester. Although five patients with porencephaly/PVI and one patient with malformation had asymmetric brain lesion and more severe contralateral palsy, they showed diplegia rather than hemiplegia, suggesting the presence of undetected lesions in the pyramidal tract.

In conclusion, the present study, which to our knowledge has used the largest reported sample size of patients born at term with spastic diplegia, found that (1) 41.9% of patients had normal MRI findings, (2) there was no difference in the incidence of severe motor dysfunction and intellectual disability between patients with abnormal and normal brain MRI findings, and (3) patients with no brain abnormalities exhibited significantly fewer epileptic episodes than those with abnormal MRI findings. Spastic diplegia in patients born at term with no brain abnormalities may be the result of unidentified pathophysiology that requires further clarification. The investigation of possible genetic or early prenatal factors may provide a better understanding of the pathogenesis of spastic diplegia in those born at term.

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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 severe 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 multigenic 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 *MTHFD1L* (15). Another component of mitochondrial FOCM, the glycine cleavage system (GCS), acts to break down glycine to donate one-carbon units to tetrahydrofolate (THF), generating 5,10-methylenetetrahydrofolate (methylene-THF; Fig. 1B) (16,17). The GCS consists of four enzyme components, each of which is required for the glycine cleavage reaction (18,19). The components—glycine dehydrogenase (decarboxylating) (GLDC; P-protein), aminomethyltransferase (AMT; T-protein), glycine cleavage system protein H (GCSH; H-protein) and dihydrolipoamide dehydrogenase (DLD; L-protein)—are encoded by distinct genes: *GLDC*, *AMT*, *GCSH* and *DLD*, respectively. The functions of *GLDC*, *AMT* and *GCSH* are specific to the GCS, whereas *DLD* encodes a housekeeping enzyme. GCS components

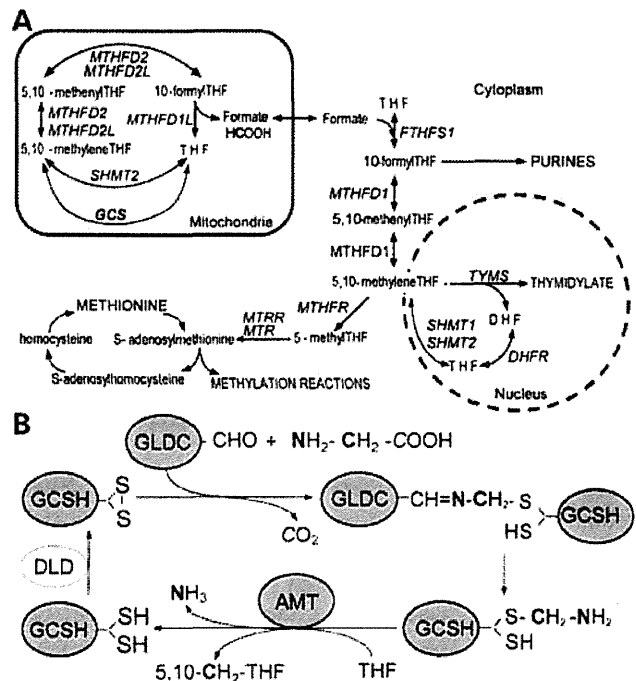


Figure 1. Schematic diagrams summarizing the key reactions of folate-mediated one-carbon metabolism and the GCS. (A) Folates donate and accept one-carbon units in the synthesis of purines, thymidylate and methionine. Mitochondrial FOCM supplies one-carbon units to the cytoplasm via formate. The GCS is a key component of mitochondrial FOCM that breaks down glycine and generates 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 CO_2 and NH_3 , 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 CO_2 .

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 ($n = 22$).

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		Number of mutation carriers in the Japanese cohort		Number of mutation carriers in the Swedish cohort		Variant <i>GLDC</i> enzyme activity ^a
			NTD group (type ^b) (n = 166) ^c	Control group (n = 189) ^c	NTD group (type ^b) (n = 14) ^c	Control group (n = 36) ^c	NTD group (type ^b) (n = 76) ^c	Control group (n = 145) ^c	
<i>AMT</i>									
Exon 2	c.103A>C	p.R35R	0	1	0	0	0	—	
	c.214A>G	p.T72A	0	0	0	1	0	—	
Exon 6	c.623C>A	p.A208D	0	2	0	0	0	—	
	c.631G>A	p.E211K ^d	2 (SBA)	0	0	0	1	—	
Exon 7	c.589G>C	p.D197H	0	0	1 (An)	0	0	—	
	c.825T>A	p.N275K	0	1	0	0	0	—	
	c.850G>C	p.V284L	1 (SBA)	0	0	0	0	—	
<i>GLDC</i>									
Exon 1	c.52G>T	p.G18C	2 (SBO/SBA)	2	0	0	2 (SBA)	2	84%
Exon 5	c.668C>G	p.P223R	0	0	0	1	0	—	92%
Exon 12	c.1508A>C	p.E503A	1 (SBA)	0	0	0	0	0	—
	c.1512G>C	p.E504D	1 (SBA)	0	0	0	0	0	99%
	c.1519G>C	p.G507R	1 (An)	0	0	0	0	0	17%
	c.1525C>G	p.P509A ^e	1 (An)	0	0	0	0	0	41%
Exon 14	c.1550G>C	p.S517T	0	0	0	0	1 (SBA)	0	—
	c.1570G>C	p.V524L	1 (SBA)	0	0	0	0	0	34%
	c.1705G>A	p.A569T ^f	3 (SBA/SBO/SBO)	1	0	0	1 (SBA)	0	40%
	Exon 17	c.1953T>C	p.H651H	0	1	0	0	0	—
	Exon 19	c.2203G>T	p.V735L	0	2	0	0	0	—
Intron 19	c.2316-1G>A	splice	1 (SBA)	0	0	0	0	—	81%
Exon 20	c.2380G>A	p.A794T	2 (SBASBA)	0	0	0	2 (SBA)	2	88%
	c.2406G>A	p.A802A	1 (An)	0	0	0	0	0	—
Exon 21	c.2474G>A	p.G825D	0	0	1 (An)	0	0	—	24%
	c.2487C>T	p.A829A	0	1	0	0	0	—	—
Exon 23	c.2565A>C	p.A855A	1 (An)	0	0	0	0	—	—
	c.2746C>T	p.L916L	1 (Cm)	0	0	0	0	—	—
Exon 25	c.2964G>A	p.R988R	0	0	0	0	1 (SBA)	0	—
	c.2965A>G	p.I989V	0	1	0	0	0	0	130%
<i>GCSH</i>									
Exon 1	c.53C>T	p.A18V	1 (An)	1	0	0	—	—	

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.660C>T (p.L220L, rs2228095), c.666T>C (p.D222D, rs12004164), c.671G>A (p.R224H, rs28617412) and c.1384C>G (p.L462V, rs73400312); and for *GCSH*: c.62T>C (p.S21L, rs8052579), c.90C>G (p.P30P, rs8177847), c.159C>T (p.F53F, rs177876), c.218A>G (N73S, rs8177876), c.252T>C (Y84Y, rs8177907) and c.261C>G (L87L, rs8177908). Grey shading indicates loss-of-function mutations, based on enzymatic activity in the *in vitro* expression study or splicing defect.

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

^bSBA, spina bifida aperta; SBO, spina bifida occulta; An, anencephaly; Cm, craniorachischisis.

^cTotal number of UK, Japanese or Swedish NTD patients.

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

^eA 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).

^fp.A569T has previously been reported as a pathogenic mutation in a patient with typical NKH (21).

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>A). The

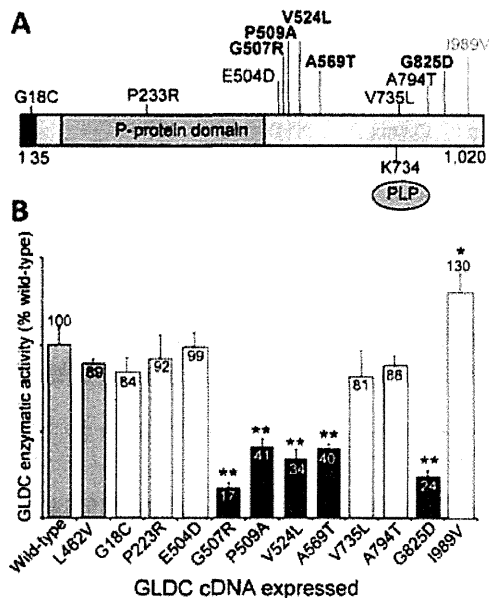


Figure 2. Characterization of *GLDC* missense mutations identified through DNA sequence analysis. (A) The schematic represents the 1020 amino acid residue *GLDC* polypeptide with the positions of the identified missense variants indicated. Mutations conferring significantly reduced activity (B) are indicated in bold. The leader peptide for mitochondrial import (shaded black) and the lysine 754-binding site for the co-factor pyridoxal phosphate (PLP) are indicated (49). (B) Enzymatic activity of *GLDC* missense variants. Expression vectors with wild-type and mutant *GLDC* cDNAs were transfected into COS7 cells for the evaluation of *GLDC* activity, which is expressed as relative activity (%) of cells expressing wild-type cDNA (shaded grey). The L462V *GLDC* enzyme (shaded grey) was tested as an example of a normally occurring variant (rs73400312). Variant proteins whose activities were significantly diminished compared with wild-type are indicated by black shading. The I989V variant, identified in a control parent, showed significantly elevated activity. Values are given as mean \pm SD of triplicate experiments (* $P < 0.05$; ** $P < 0.01$, compared with wild-type).

latter is deduced to abolish normal splicing of the *GLDC* mRNA, with predicted skipping of exon 19 resulting in loss of the reading frame. Among the 15 missense variants identified in *GLDC*, 5 were unique to the NTD group, being absent from all 562 control individuals as well as from the SNP databases. A further three novel variants were found only in controls, whereas the remainder were found in both NTDs and controls, and included previously reported SNPs.

We investigated the possible functional effects of *GLDC* missense variants by expressing wild-type and mutant cDNA constructs in COS7 cells, followed by enzymatic assay of *GLDC* activity involving a decarboxylation reaction using [14 C]glycine (22). Twelve *GLDC* variants were tested, including those that were unique to NTD patients and, therefore, hypothesized to be potentially pathogenic (Fig. 2). The L462V variant, which corresponds to a known SNP (rs73400312), was included as an example of a known normally occurring form. Five of the missense changes, G507R, P509A, V524L, A569T and G825D, resulted in a significant reduction in *GLDC* activity compared with the wild-type protein ($P < 0.001$). Notably, all five of these deleterious variants were present solely in NTD cases, whereas none of the variants that were unique to controls (P223R, V735L and I989V) showed loss of

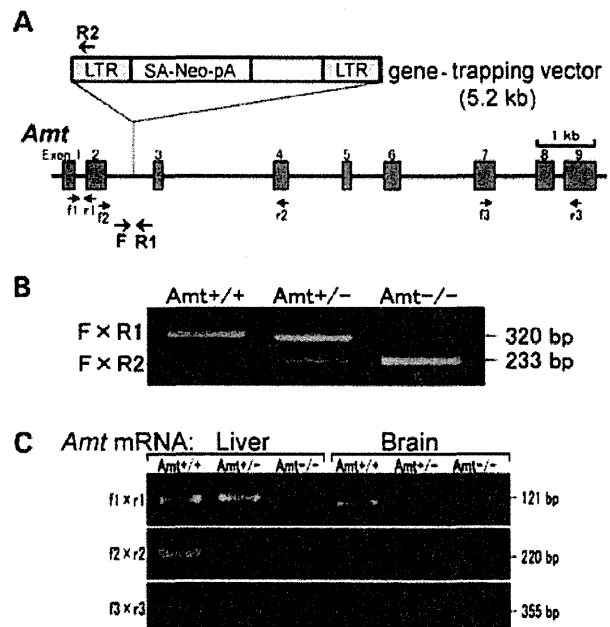


Figure 3. Generation of *Amt* knockout mouse by gene trapping. (A) The location of the gene-trap vector in *Amt* intron 2 in the ES cell line OST181110 was determined by inverse PCR. Mice carrying this mutation were generated using standard methods of blastocyst microinjection with OST181110 ES cells to generate 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 wild-type allele, whereas a 233 bp fragment 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' to the insertion site produced 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.

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 trapping expression of *Amt* (*Amt*⁻) was confirmed by RT-PCR analysis (Fig. 3). Heterozygous *Amt*^{+/-} mice were viable and fertile and exhibited no obvious malformations. Homozygous *Amt*^{-/-} mice were not observed among post-natal litters from heterozygote intercrosses, and so fetuses were examined at embryonic day (E) 17.5. Strikingly, 87% of *Amt*^{-/-} fetuses (34 out of 39) exhibited NTDs, whereas no malformations were observed in *Amt*^{+/+} ($n = 33$) or *Amt*^{+/-}

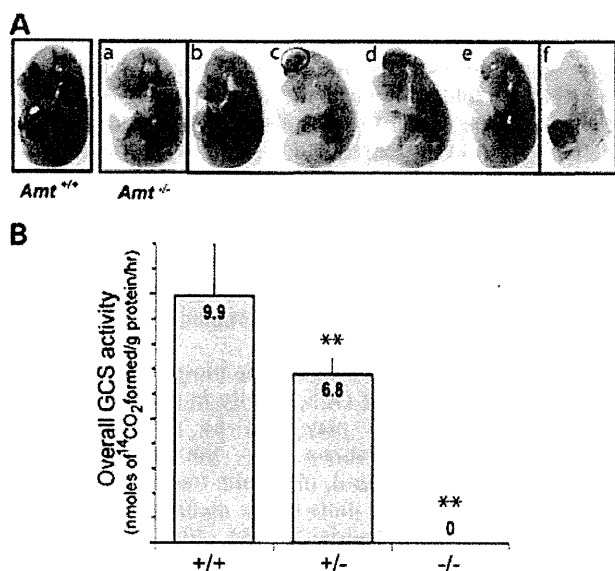


Figure 4. Mice lacking GCS activity exhibit NTDs. (A) Phenotypes of *Amt* mutant mice. NTDs were evident in the majority (88%) of *Amt*^{-/-} fetuses (examples shown are at E17.5). Various types of NTDs were observed in *Amt*^{-/-} fetuses, which principally affected the cranial region; a, no NTDs; b, small exencephaly (dotted circle); c–e, large exencephaly; f, craniorachischisis. (B) Enzymatic activity of the GCS in *Amt* knockout mice. *Amt*^{+/-} and *Amt*^{-/-} fetuses had significantly lower GCS activity in the liver than *Amt*^{+/+} fetuses, with activity in *Amt*^{-/-} samples below the level of detection (***P* < 0.01 compared with *Amt*^{+/+}).

(*n* = 66) fetuses. Defects mainly comprised exencephaly (82%), in which the cranial neural folds persistently failed to close (Fig. 4). There was also a low frequency of the more severe condition, craniorachischisis (5%), in which the neural tube remains open from the mid- and hindbrain, and throughout the spinal region (Fig. 4). Fetal liver samples were subjected to enzyme assay to determine overall activity of the GCS. In *Amt*^{-/-} mice, overall GCS activity was effectively ablated being below the detection level of the assay (0.01 nmoles of ¹⁴C₂ formed/gram protein/h), consistent with the *Amt*⁻ allele being a functional null (22) (Fig. 4). These findings confirm that *AMT* function is essential for GCS activity, and that the latter is necessary for successful neural tube closure.

Given that GCS is a component of FOCM (Fig. 1), we evaluated the possible prevention of NTDs by folate-related metabolites. Maternal supplementation was performed with folic acid, thymidine monophosphate (TMP), methionine or methionine plus TMP (23). Neither folic acid nor TMP significantly affected the frequency of NTDs among the homozygous *Amt*^{-/-} offspring. However, we observed a significant protective effect of maternal supplementation with methionine or methionine plus TMP, compared with the non-treated group (*P* < 0.05; Fig. 5).

DISCUSSION

NTDs remain among the commonest human birth defects and understanding their genetic basis presents a considerable

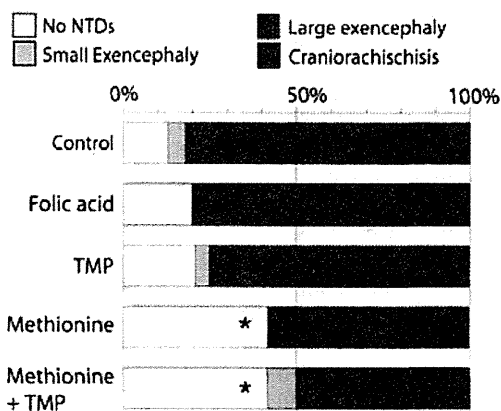


Figure 5. Maternal supplementation of *Amt* mutant embryos with folic acid, TMP or methionine. Maternal treatment with folic acid (*n* = 10 homozygous mutant fetuses) or TMP (*n* = 12) had no significant effect on NTD frequency, whereas the frequency of unaffected embryos was significantly increased following treatment with methionine (*n* = 12) or methionine plus TMP group (*n* = 12). The asterisk indicates significant difference compared with non-treated group (*P* < 0.05).

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. 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.

Where parental samples were available (6 of the 11 NTD cases that involved putative mutations in *GLDC*), we demonstrated parent-to-child transmission (Supplementary Material, 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).

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

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 estimate 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 *Folr1* 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 (*spotch*) 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 *spotch* (*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 that ~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 [$1\text{-}^{14}\text{C}$]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*