Eur J Pediatr (2012) 171:301–305
DOI 10.1007/s00431-011-1534-2

ORIGINAL PAPER

Proportion of malformations and genetic disorders among cases encountered at a high-care unit in a children's hospital

Akiko Soneda · Hideki Teruya · Noritaka Furuya · Hiroshi Yoshihashi · Keisuke Enomoto · Aki Ishikawa · Kiyoshi Matsui · Kenji Kurosawa

Received: 5 May 2011 / Accepted: 5 July 2011 / Published online: 16 July 2011

Abstract Genetic disorders and birth defects account for a high percentage of the admissions in children's hospitals. Congenital malformations and chromosomal abnormalities are the most common causes of infant mortality. So their effects pose serious problems for perinatal health care in Japan, where the infant mortality is very low. This paper describes the reasons for admissions and hospitalization at the high-care unit (HCU) of a major tertiary children's referral center in Japan. We retrospectively reviewed 900 admission charts for the period 2007–2008 and found that genetic disorders and malformations accounted for a significant proportion of the cases requiring admission to the HCU. Further, the rate of recurrent admission was higher for patients with genetic disorders and malformations than for those with acquired, non-genetic conditions. Over the past 30 years, admissions attributed to genetic disorders and malformations has consistently impacted on children's hospital and patients with genetic disorders and malformations form a large part of this facility. These results reflect improvements in medical care for patients with genetic disorders and malformations and further highlight the large proportion of cases with genetic disorders, for which highly specialized management is required. Moreover, this study emphasizes the need for involvement of clinical geneticists in HCUs at children's hospitals.

Keywords Malformation · Genetic disease · High-care unit · Children's hospital · Mortality

Grant sponsor The Ministry of Health, Labor and Welfare, Japan

A. Soneda · N. Furuya · H. Yoshihashi · K. Enomoto · A. Ishikawa · K. Kurosawa (✉)
Division of Medical Genetics,
Kanagawa Children's Medical Center,
2-138-4 Mutsukawa, Minami-ward,
Yokohama 232-8555, Japan
e-mail: kkurosawa@kcmc.jp

H. Teruya
Division of Critical Care Medicine,
Kanagawa Children's Medical Center,
Yokohama, Japan

K. Matsui
Division of General Pediatrics,
Kanagawa Children's Medical Center,
Yokohama, Japan

H. Teruya
Department of Pediatrics, Yokohama Rosai Hospital,
Yokohama, Japan

K. Kurosawa
Clinical Research Institute, Kanagawa Children's Medical Center,
Yokohama, Japan

Introduction

Genetic disorders and birth defects account for a high percentage of the admissions to children's hospitals [4, 13]. In 2008 [5], the Ministry of Health, Labor and Welfare in Japan reported that congenital malformations, chromosomal abnormalities, and genetic diseases are the leading causes of death in children during the first year of life. As per that report, 999 infants under the age of 1 year died of congenital malformations and chromosomal abnormalities; this corresponds to 35.7% of the total number of deaths in this age group. Since 1985, congenital malformations and chromosomal abnormalities have remained the leading causes of infant mortality in Japan [5]. Indeed, in USA it

has been found that patients with genetic disorders had a greater need for hospital admission and were hospitalized for longer durations than were those without genetic disorders [14].

However, recent advances in treatment are likely to improve the survival of individuals with congenital malformations, which, in turn, is likely to increase the rates of readmission to pediatric intensive care units (PICUs) [16]. Several studies have assessed the role of genetic disorders in pediatric mortality and hospitalization [2, 6, 7, 16]. Congenital malformations and chromosomal abnormalities pose serious challenges for perinatal health care in this country, as they are the leading contributors to the infant mortality rate in Japan.

In this study, we assessed the reasons for admissions and hospitalization to the high-care unit (HCU) of a major tertiary children's referral center in Kanagawa Prefecture, Japan, and compared our findings to those of a study of this unit 30 years ago. To elucidate the impact and contribution of birth defects and genetic diseases on pediatric hospitalization, we studied the reason for hospitalization, underlying diagnoses, and duration of hospitalization in this children's hospital in Japan.

Materials and methods

Permission for the study was obtained from the Ethical Committee of our medical center.

We retrospectively analyzed the cases of children hospitalized at the HCU of Kanagawa Children's Medical Center (KCMC) between June 2007 and December 2008. KCMC is a major tertiary children's referral center for pediatric cardiology, surgery, and cancer cases and serves a large area in Kanagawa Prefecture, Japan. It has an institute for the severely handicapped, a PICU, a neonatal intensive care unit, and an HCU. In contrast to the PICU, which admits patients who have undergone cardiovascular or neurosurgery, the HCU specializes in pediatric patients with other acute conditions. All of the patients were included if they were admitted to the HCU from the emergency room, operating room, or inpatient ward. KCMC, with 419 beds, is the only specialized pediatric hospital in Kanagawa Prefecture, where the total number of births is 80,000 annually [8, 9]. About 8,500 patients (male/female, 1:1) were admitted to KCMC in 2007, and the average of hospital stay was 15.3 days.

We summarized and reviewed the medical charts of all patients admitted to the HCU. The charts and summaries were reviewed for age, sex, duration of hospitalization, underlying disease, and reason for admission. Subcategories were created for the underlying diseases and reason for admission.

The underlying disease was classified into two main categories: genetic conditions and acquired (non-genetic) conditions. Genetic conditions were considered to include chromosomal abnormalities, recognizable malformation and dysplasia, multiple malformations, isolated malformations (e.g., those related to the heart, central nervous system (CNS), and respiratory and gastrointestinal tracts), other single-gene defect-related conditions, mitochondrial diseases, and metabolic disorders (Table 1). All cases of chromosomal abnormalities and multiple malformations were examined using standard karyotyping. Cases of recognizable malformation/dysplasia were ascertained by clinical dysmorphologists (H.Y., N.F., and K.K.). Acquired conditions were considered to include perinatal complications, trauma, neoplasm, and sequelae of severe infectious conditions.

The reasons for admission were classified as problems of the respiratory system, CNS, heart, gastrointestinal tract, kidneys and urinary tract, infectious diseases, post-operative management, and unknown condition. Those cases that did not fall into these categories were placed into a category called "others."

Statistical analyses were performed to compare the duration of hospitalization and the age distribution, using StatView version 5.0 (SAS Institute, Inc; Cary, NY). Categorical data were reported as counts and percentages, and continuous data as mean (SD) or median values. Statistical differences for categorical variables were determined by using chi-squared analyses. Median differences were compared by Mann–Whitney *U* test.

Results

A total of 900 admissions, consisting of 687 individual cases with 200 recurrent admissions, were reviewed. Sixteen admissions were excluded from the study because of insufficient information regarding the underlying causes for admission.

The median age at admission was 3.5 years (range, 1 day–32.5 years), and the sex ratio was 1.36 (396 males and 291 females). The median lengths of hospitalization in the HCU were 4 days. Table 2 shows the distribution of the 884 admissions across the different categories of causes for admission. Most patients were admitted for common medical problems, including respiratory problems, post-operative management, and CNS problems. Of the 298 admissions for respiratory problems, most cases involved respiratory infection, including pneumonia and bronchitis. Admissions for post-operative management accounted for 30.7% cases (271 of 884 admissions), while CNS problems such as convulsions, encephalitis, and meningitis accounted for 16.3% (144 of 884 admissions).

Table 1 Definitions of categories

Category	Examples
Chromosomal syndromes	Down syndrome, trisomies 13 and 18, cri du chat syndrome, and Wolf–Hirschhorn syndrome
Recognizable malformation/dysplasia	22q11.2 deletion syndrome, CHARGE syndrome, and VATER association, Lowe syndrome, achondroplasia, Crouzon syndrome, Noonan syndrome, and Treacher–Collins syndrome
Multiple malformations	
Isolated malformations	
Congenital heart diseases	VSD ASD, AVSD, TGA, and DORV
Central nervous system malformations	Schistorrhachis, hydrocephalus, and meningoencephalocele
Gastrointestinal malformations	Diaphragmatic hernia, biliary atresia, and congenital intestinal obstruction
Respiratory system malformations	CCAM and tracheal stenosis
Other isolated malformations	Cleft palate and cleft lip
Single-gene defect	Metabolic diseases, spinal muscular atrophy, and spinocerebellar degeneration
Mitochondrion	

The classification of the underlying conditions of the 687 patients is shown in Table 3. In 13 cases, the data for identifying the underlying disease were insufficient (e.g., charts were missing). These cases were categorized as "unknown condition." Of the total 687 patients, 372 (54.1%) had genetic disorders and the remaining 302 (44.0%) had acquired conditions unrelated to genetic disorders, including perinatal complications, neoplasm, and trauma. Among the 372 patients with genetic disorders, 72 had chromosomal abnormalities, with Down syndrome (29 cases) being the most common underlying disorder. Seventy patients had recognizable malformations and dysplasia, with conditions such as osteogenesis imperfecta, 22q11.2 deletion syndromes, CHARGE syndrome, and VATER association. Multiple malformations with unrecognizable patterns were present in 38 cases while isolated malformations, including CNS malformation, congenital heart disease, and gastrointestinal malformation were present in 160 cases.

We also summarized the reasons for the total of 884 admissions, according to the underlying condition (genetic or acquired). Of these admissions, 200 were readmissions. Patients with genetic disorders and malformations had a greater tendency to be hospitalized repeatedly as compared with those with acquired conditions (Fig. 1). In both genetic and acquired condition categories, respiratory disease, post-operative management, and CNS problems were the major medical problems leading to admission.

We further compared age distribution and the lengths of hospitalization between the groups with genetic and acquired disorders (Table 4). The patients with genetic

Table 2 Medical problems for admission (N=884)

Causes for admission	Number	Percent
Respiratory problems	298	33.7
Post-operative management	271	30.7
CNS problems	144	16.3
Gastrointestinal problems	35	4.0
Cardiac diseases	23	2.6
Other infectious state	23	2.6
Examination	21	2.4
Kidney and urinary tract problems	14	1.6
Other	55	6.2
Total	884	100.0

Table 3 Classification of underlying diseases in 678 patients

Underlying diseases	Number	Percent
Genetic disorders and malformations (subtotal)	372	54.1
Chromosomal abnormalities	(72)	10.5
Recognizable malformation/dysplasia	(70)	10.2
Multiple malformations	(38)	5.5
Isolated malformations	(subtotal:160)	23.3
Central nervous system malformation	(71)	10.3
Congenital heart disease	(35)	5.1
Gastrointestinal malformation	(32)	4.7
Respiratory system malformation	(9)	1.3
Other isolated malformations	(13)	1.9
Single-gene defect	(26)	3.8
Mitochondrion	(6)	0.9
Acquired non-genetic conditions (subtotal)	302	44.0
Perinatal complications	(66)	9.6
Neoplasm	(38)	5.5
Trauma(non-accidental and accidental)	(27)	3.9
Infection	(16)	2.3
Other	(155)	22.6
Unknown	13	1.9
Total	687	100.0

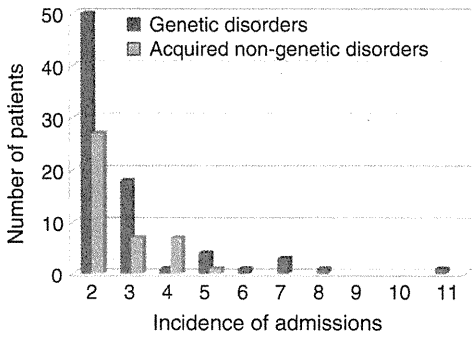

Fig. 1 Comparison of the incidence of admission between the groups with genetic disorders and acquired disorders. In both groups, a total of 200 patients were readmitted. The group with genetic disorders generally required frequent readmission

disorders were significantly younger than those with acquired conditions (median age, 2.0 vs. 4.9 years; $P<0.0001$). There is no significant difference in the length of hospitalization between the patients with genetic disorders and those with acquired conditions (median, 4 vs. 4 days; $P=0.26$), but some patients with genetic disorders had much longer hospitalization (mean, 13.0 vs. 7.0 days; $P=0.007$; range, 1–979 days). Among the reasons for admission, respiratory problems tended to have a longer duration of hospitalization for patients with genetic disorders than for those with acquired conditions (median, 7 vs. 5 days; $P=0.17$).

Discussion

Our study shows that genetic disorders and malformations account for a significant proportion of cases requiring admission to the HCU. Additionally, the rate of recurrent admission was higher among patients with genetic disorders and malformations than among those with acquired non-genetic conditions. This finding is in agreement with those of previous reports for other countries [4, 13].

Several studies from different countries have previously suggested that genetic conditions and malformations and the associated mortality and morbidity have a significant impact on the cost burden for society and the patients' families. Cunniff et al. reported that 19% of deaths in a PICU were in cases of heritable disorders [1]. Stevenson and Carey reported that the 34.4% of deaths in a children's hospital were due to malformations and genetic disorders [15]. On the basis of a population-based study, Yoon et al. reported that the overall rate of hospitalization was related to birth defects and genetic diseases, and varied with age and race/ethnicity [16]. McCandless et al. reported the enormous impact of genetic disease on inpatient pediatrics and the health care system in both admission rates and the total hospital charges [11]. These studies emphasize the importance of understanding the impact that genetic diseases have on mortality and healthcare strategies [15]. Furthermore, it is also clear that early recognition of the underlying disorders is necessary for optimal management of patients with genetic disorders.

Our study highlights another aspect related to the impact of genetic disorders and malformations. In 1981, Matsui et al. analyzed the cases of 18,736 children of total admission during 1975–1979 to KCMC and found that 44% had genetic disorders and malformations [10]. Although our study period and ward are limited to those in the HCU, the patients with genetic disorders and malformations had consistently significant impact in KCMC during the ensuing three decades. Further, it emphasizes that medical care for acute conditions and surgical procedures frequently requires highly specialized knowledge of unusual disease conditions and should be provided in consultation with specialists such as clinical geneticists.

Table 4 Comparison of patients with genetic disorder vs. acquired condition on ages at admission and lengths of stay

	Genetic disorders		Acquired conditions		
	Median (range)	*n*	Median (range)	*n*	*P*
Ages	2.0 years (1 day–27.0 years)	372*	4.9 years (9 days–32.5 years)	302*	<0.0001
Length of hospitalization (days)					
Respiratory problem	7 (1–979)	182	5 (1–97)	109	0.17
CNS	4 (1–54)	73	4 (1–207)	68	0.61
Cardiovascular	4 (2–11)	13	4 (2–24)	8	0.94
Gastrointestinal	5.5 (1–37)	22	5 (2–15)	12	0.60
Kidney and urinary tract	3 (2–12)	5	8 (2–12)	9	0.32
Sepsis	3.5 (2–9)	14	7 (2–20)	9	0.19
Post-operative care	2 (1–49)	174	2 (1–62)	93	0.18
Total	4 (1–979)	518	4 (1–207)	366	0.26

*For the patients who have recurrent admissions, the only first admission was calculated

Although the strategies for management of respiratory infection, by means of newly developed antibiotics and mechanical ventilators, and surgical intervention for infants with malformations, have improved, the general strategies for the medical treatment of genetic disorders and malformations remain to be clarified. Hall commented on the report by Yoon et al. [16] and emphasized the significance of basic research on the human genome and developmental genetics [3]. As shown in Table 2, genetic disorders and malformations include rare diseases, which, although uncommon, remain an important public-health issue and a challenge for the medical community [12].

Our study had the limitations of genetic studies and evaluation in cases with multiple malformations and other isolated malformations. The underlying conditions of most patients in this study were ascertained by clinical geneticists, but high-resolution genome analysis with arrays using comparative genomic hybridization was applied in only limited cases. Recently, research attention has focused to a large extent on rare genetic disorders and Mendelian diseases, because of their significant effect on human health, with the aim of identifying disease-related genetic variations. Re-evaluation and classification of underlying disorders, especially in the case of multiple congenital anomalies in undiagnosed patients, are required for further analysis.

Another limitation of our study is estimation of the financial burden of the group of patients with a genetic background. McCandless et al. showed that the disorders with genetic determinant account for 81% of the total hospital charges [11]. Their results are consistent with those of Hall et al. in 1978 [4]. Further analysis of financial burden in our study may provide useful information for improvement of health care systems.

In conclusion, we report here the proportion of genetic disorders and malformations among cases encountered at the HCU of a tertiary children's medical center in Japan. Over 30 years, the proportion of admissions attributed to genetic disorders and malformations has impact and currently accounts for more than half of admissions to this facility. These results firstly indicate improvements in medical care for patients with genetic disorders and malformations and further highlight the large proportion of cases with genetic disorders. As these cases require highly specialized management, the involvement of clinical geneticists in HCUs at children's hospitals is crucial. Eventually, a better fundamental understanding of genetic disorders and malformations may lead to further improvements in medical care and may reduce the impact of these conditions on the patients and their families.

Acknowledgments The authors are grateful to Dr. Hiroyuki Ida (Tokyo Jikei University) for his valuable comments. This research was supported in part by a grant-in-aid from the Ministry of Health, Labor and Welfare, Japan.

Conflict of interest The authors declare no conflict of interest.

References

1. Cunniff C, Carmack JL, Kirby RS, Fiser DH (1995) Contribution of heritable disorders to mortality in the pediatric intensive care unit. Pediatrics 95:678–681
2. Garrison MM, Jeffries H, Christakis DA (2005) Risk of death for children with Down syndrome and sepsis. J Pediatr 147:748–752
3. Hall JG (1997) The impact of birth defects and genetic diseases. Arch Pediatr Adolesc Med 151:1082–1083
4. Hall JG, Poweres EK, McIlvaine RT, Ean VH (1978) The frequency and financial burden of genetic disease in a pediatric hospital. Am J Med Genet 1:417–436
5. Health and Welfare Statistics Association (2010) Mortality rate of infant. J Health Welfare Statist 57:63–65, in Japanese
6. Heron M, Sutton PD, Xu J, Vetura SJ, Strobino DM, Guyer B (2010) Annual summary of vital statistics: 2007. Pediatrics 125:4–15
7. Hudome SM, Kirby RS, Senner JW, Cunniff C (1994) Contribution of genetic disorders to neonatal mortality in a regional intensive care setting. Am J Perinatol 11:100–103
8. Kuroki Y, Konishi H (1984) Current status and perspectives in the Kanagawa Birth Defects Monitoring Program (KAMP). Cong Anom 24:385–393
9. Kurosawa K, Imaizumi K, Masuno M, Kuroki Y (1994) Epidemiology of limb-body wall complex in Japan. Am J Med Genet 51:143–146
10. Matsui I, Naito K, Hanawa Y et al (1981) Impact of the congenital birth defects on children's health care. J Jpn Pediatr Soc 85:889–897, in Japanese
11. McCandless SE, Brunger JW, Cassidy SB (2004) The burden of genetic disease on inpatient care in a children's hospital. Am J Hum Genet 74:121–127
12. Schieppati A, Henter J-I, Daina E, Aperia A (2008) Why rare diseases are an important medical and social issue. Lancet 371:2039–2041
13. Scriver CR, Neal JL, Saginur R, Clow A (1973) The frequency of genetic disease and congenital malformation among patients in a pediatric hospital. Can Med Assoc J 108:1111–1115
14. Sever L, Lynberg MC, Edmonds LD (1993) The impact of congenital malformations on public health. Teratology 48:547–549
15. Stevenson DA, Carey JC (2004) Contribution of malformations and genetic disorders to mortality in a children's hospital. Am J Med Genet 126A:393–397
16. Yoon PW, Olney RS, Khoury MJ, Sappenfield WM, Chavez GF, Taylor D (1997) Contribution of birth defects and genetic diseases to pediatric hospitalizations. Arch Pediatr Adolesc Med 151:1096–1103

HMG Advance Access published December 30, 2011

Human Molecular Genetics, 2011 **1–8**
doi:10.1093/hmg/ddr585

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

Ayumi Narisawa[1,2], Shoko Komatsuzaki[1], Atsuo Kikuchi[3], Tetsuya Niihori[1], Yoko Aoki[1], Kazuko Fujiwara[4], Mitsuyo Tanemura[5], Akira Hata[6], Yoichi Suzuki[6], Caroline L. Relton[7], James Grinham[8], Kit-Yi Leung[8], Darren Partridge[8], Alexis Robinson[8], Victoria Stone[8], Peter Gustavsson[9], Philip Stanier[8], Andrew J. Copp[8], Nicholas D.E. Greene[8,*], Teiji Tominaga[2], Yoichi Matsubara[1] and Shigeo Kure[1,3,*]

[1]Department of Medical Genetics, [2]Department of Neurosurgery and [3]Department of Pediatrics, Tohoku University School of Medicine, Sendai, Japan, [4]Institute for Enzyme Research, University of Tokushima, Tokushima, Japan, [5]Tanemura Women's Clinic, Nagoya, Japan, [6]Department of Public Health, Chiba University School of Medicine, Chiba, Japan, [7]Human Nutrition Research Centre, Institute for Ageing and Health, Newcastle University, Newcastle upon Tyne, UK, [8]Institute of Child Health, University College London, London, UK and [9]Department of Molecular Medicine and Surgery, Karolinska Institute, Stockholm, Sweden

Received October 26, 2011; Revised November 25, 2011; Accepted December 6, 2011

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

*To whom correspondence should be addressed at: Neural Development Unit, UCL Institute of Child Health, Guilford Street, London, WC1N 1EH, UK. Email: n.greene@ucl.ac.uk (N.D.E.G.); Department of Pediatrics, Tohoku University School of Medicine, 1-1 Seiryomachi, Aobaku, Sendai 980-8574, Japan. Email: kure@med.tohoku.ac.jp (S.Ku.)

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

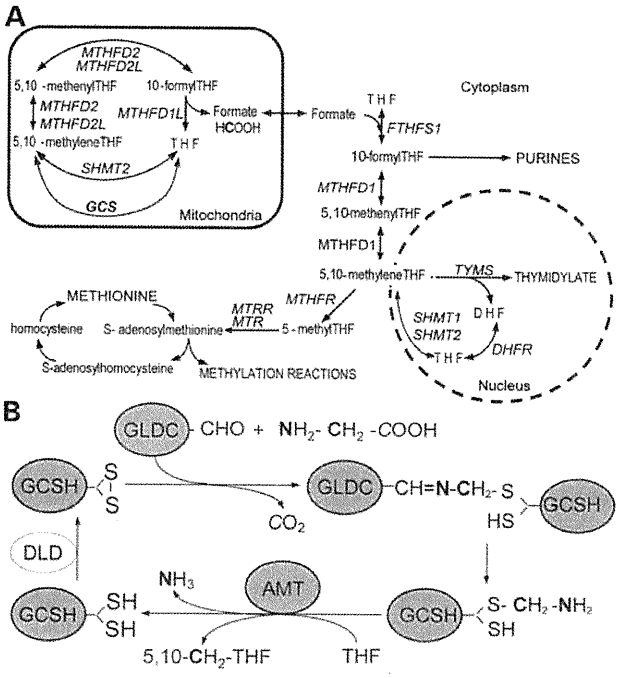

Figure 1. Schematic diagrams summarizing the key reactions of folate-mediated one-carbon metabolism and the GCS. (**A**) Folates donate and accept one-carbon units in the synthesis of purines, thymidylate and methionine. Mitochondrial FOCM supplies one-carbon units to the cytoplasm via formate. The GCS 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.

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 GLDC 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]	
AMT									
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	—	
	c.589G>C	p.D197H	0	0	1 (An)	0	0	—	
Exon 7	c.825T>A	p.N275K	0	1	0	0	0	—	
	c.850G>C	p.V284L	1 (SBA)	0	0	0	0	—	
GLDC									
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%
	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%
Exon 14	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	—	81%
Intron 19	c.2316-1G>A	splice	1 (SBA)	0	0	0	0	—	
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	—	—
	c.2565A>C	p.A855A	1 (An)	0	0	0	0	—	—
Exon 23	c.2746C>T	p.L916L	1 (Crn)	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%
GCSH									
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.

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

[b]SBA, spina bifida aperta; SBO, spina bifida occulta; An, anencephaly; Crn, craniorachischisis.

[c]Total number of UK, Japanese or Swedish NTD patients.

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

[e]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).

[f]p.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

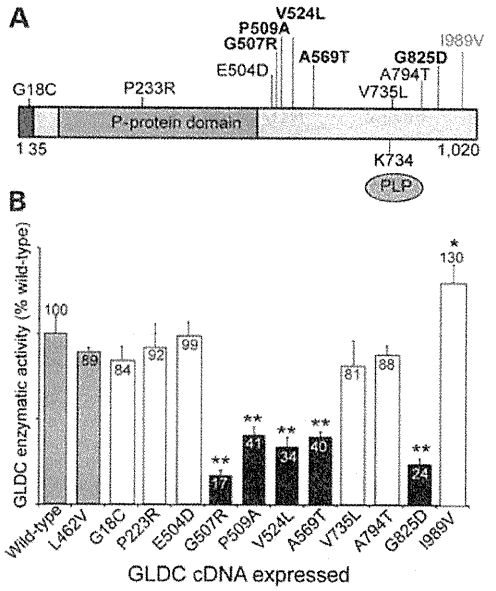

Figure 2. Characterization of *GLDC* missense mutations identified through DNA sequence analysis. (**A**) The schematic represents the 1020 amino acid residue GLDC polypeptide with the positions of the identified missense variants indicated. Mutations conferring significantly reduced activity (**B**) 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 ± SD of triplicate experiments ($^{*}P < 0.05$; $^{**}P < 0.01$, compared with wild-type).

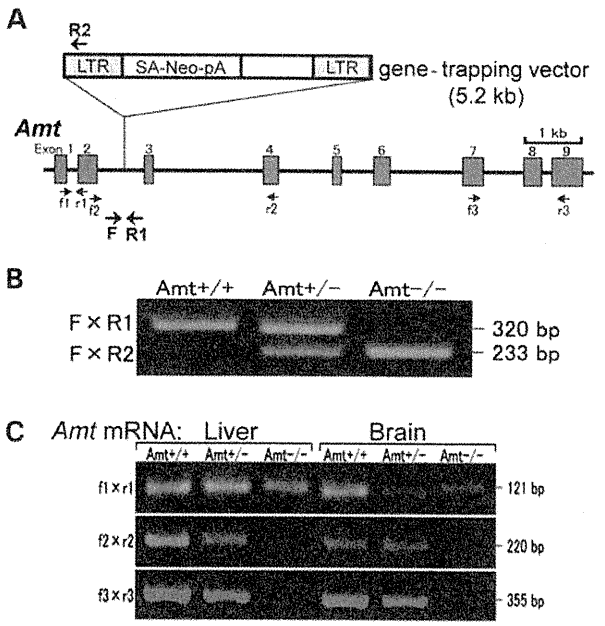

Figure 3. Generation of *Amt* knockout mouse by gene trapping. (**A**) The location of the gene-trap vector in *Amt* intron 2 in the ES cell line OST181110 was determined by inverse PCR. Mice carrying this mutation were generated using standard methods of blastocyst microinjection with OST181110 ES cells 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.

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 [1-^{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 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 $Amt^{+/-}$

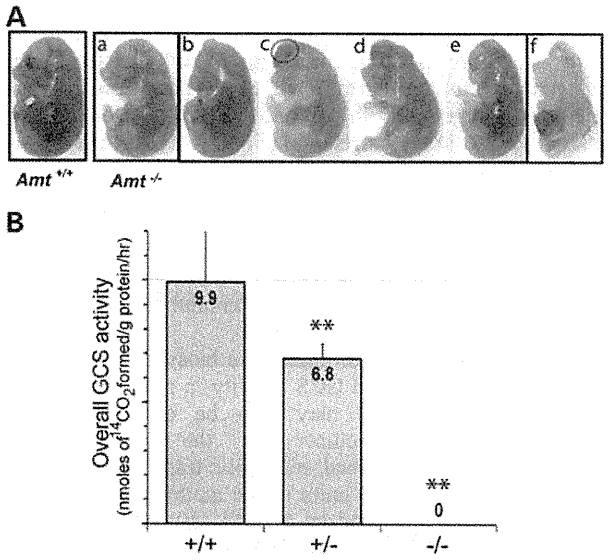

Figure 4. Mice lacking GCS activity exhibit NTDs. (**A**) Phenotypes of *Amt* mutant mice. NTDs were evident in the majority (88%) of $Amt^{-/-}$ fetuses (examples shown are at E17.5). Various types of NTDs were observed in $Amt^{-/-}$ fetuses, which principally affected the cranial region; a, no NTDs; 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 $Amt^{+/+}$).

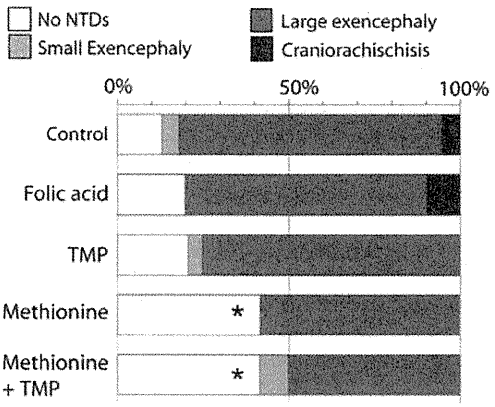

Figure 5. Maternal supplementation of *Amt* mutant embryos with folic acid, TMP or methionine. Maternal treatment with folic acid ($n = 10$ homozygous mutant fetuses) or TMP ($n = 12$) had no significant effect on NTD frequency, whereas the frequency of unaffected embryos was significantly increased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

($n = 66$) 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 $^{14}CO_2$ 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 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 (*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 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-^{14}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

enzymatic activity was determined, in triplicate, by exchange reaction between carbon dioxide and glycine using $NaH^{14}CO_3$ 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.

FUNDING

This work was supported by a research grant from the Ministry of Education, Culture, Sports, Science and Technology and a Research Grant from the Ministry of Health, Labour and Public Welfare in Japan. Research at Newcastle University was funded by the Newlife Foundation. Research at UCL Institute of Child Health was supported by SPARKS, the Wellcome Trust, Medical Research Council, UCL Biomedical Research Centre and by Great Ormond Street Hospital Children's Charity. Funding to pay the Open Access publication charges for this article was provided by Wellcome Trust.

REFERENCES

1. Greene, N.D. and Copp, A.J. (2009) Development of the vertebrate central nervous system: formation of the neural tube. *Prenatal Diag.*, **29**, 303–311.
2. Harris, M.J. and Juriloff, D.M. (2007) Mouse mutants with neural tube closure defects and their role in understanding human neural tube defects. *Birth Defects Res. A Clin. Mol. Teratol.*, **79**, 187–210.
3. Greene, N.D.E., Stanier, P. and Copp, A.J. (2009) Genetics of human neural tube defects. *Hum. Mol. Genet.*, **18**, R113–R129.
4. Au, K.S., Ashley-Koch, A. and Northrup, H. (2010) Epidemiologic and genetic aspects of spina bifida and other neural tube defects. *Dev. Disabil. Res. Rev.*, **16**, 6–15.
5. Harris, M.J. and Juriloff, D.M. (2010) An update to the list of mouse mutants with neural tube closure defects and advances toward a complete genetic perspective of neural tube closure. *Birth Defects Res. A Clin. Mol. Teratol.*, **88**, 653–669.
6. Copp, A.J. and Greene, N.D.E. (2010) Genetics and development of neural tube defects. *J. Pathol.*, **220**, 217–230.
7. Blom, H.J., Shaw, G.M., Den Heijer, M. and Finnell, R.H. (2006) Neural tube defects and folate: case far from closed. *Nat. Rev. Neurosci.*, **7**, 724–731.
8. Molloy, A.M., Brody, L.C., Mills, J.L., Scott, J.M. and Kirke, P.N. (2009) The search for genetic polymorphisms in the homocysteine/folate pathway that contribute to the etiology of human neural tube defects. *Birth Defects Res. A Clin. Mol. Teratol.*, **85**, 285–294.
9. Beaudin, A.E. and Stover, P.J. (2009) Insights into metabolic mechanisms underlying folate-responsive neural tube defects: a mini review. *Birth Defects Res. A Clin. Mol. Teratol.*, **85**, 274–284.
10. Tibbetts, A.S. and Appling, D.R. (2010) Compartmentalization of mammalian folate-mediated one-carbon metabolism. *Annu. Rev. Nutr.*, **30**, 57–81.
11. Boyles, A.L., Hammock, P. and Speer, M.C. (2005) Candidate gene analysis in human neural tube defects. *Am. J. Med. Genet. C Semin. Med. Genet.*, **135**, 9–23.
12. Shaw, G.M., Lu, W., Zhu, H., Yang, W., Briggs, F.B., Carmichael, S.L., Barcellos, L.F., Lammer, E.J. and Finnell, R.H. (2009) 118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects. *BMC Med. Genet.*, **10**, 49.
13. Martinez, C.A., Northrup, H., Lin, J.I., Morrison, A.C., Fletcher, J.M., Tyerman, G.H. and Au, K.S. (2009) Genetic association study of putative functional single nucleotide polymorphisms of genes in folate metabolism and spina bifida. *Am. J. Obstet. Gynecol.*, **201**, 394–411.
14. Dunlevy, L.P.E., Chitty, L.S., Doudney, K., Burren, K.A., Stojilkovic-Mikic, T., Stanier, P., Scott, R., Copp, A.J. and Greene, N.D.E. (2007) Abnormal folate metabolism in foetuses affected by neural tube defects. *Brain*, **130**, 1043–1049.
15. Parle-McDermott, A., Pangilinan, F., O'Brien, K.K., Mills, J.L., Magee, A.M., Troendle, J., Sutton, M., Scott, J.M., Kirke, P.N., Molloy, A.M. and Brody, L.C. (2009) A common variant in MTHFD1L is associated with neural tube defects and mRNA splicing efficiency. *Hum. Mutat.*, **30**, 1650–1656.
16. Kikuchi, G. (1973) The glycine cleavage system: composition, reaction mechanism, and physiological significance. *Mol. Cell. Biochem.*, **1**, 169–187.
17. Kure, S., Tada, K. and Narisawa, K. (1997) Nonketotic hyperglycinemia: biochemical, molecular, and neurological aspects. *Jpn J. Hum. Genet*, **42**, 13–22.
18. Kure, S., Narisawa, K. and Tada, K. (1992) Enzymatic diagnosis of nonketotic hyperglycinemia with lymphoblasts. *J. Pediatr.*, **120**, 95–98.
19. Hayasaka, K., Tada, K., Kikuchi, G., Winter, S. and Nyhan, W.L. (1983) Nonketotic hyperglycinemia: two patients with primary defects of P-protein and T-protein, respectively, in the glycine cleavage system. *Pediatr. Res.*, **17**, 967–970.
20. Ichinohe, A., Kure, S., Mikawa, S., Ueki, T., Kojima, K., Fujiwara, K., Iinuma, K., Matsubara, Y. and Sato, K. (2004) Glycine cleavage system in neurogenic regions. *Eur. J. Neurosci.*, **19**, 2365–2370.
21. Toone, J.R., Applegarth, D.A., Kure, S., Coulter-Mackie, M.B., Sazegar, P., Kojima, K. and Ichinohe, A. (2002) Novel mutations in the P-protein (glycine decarboxylase) gene in patients with glycine encephalopathy (non-ketotic hyperglycinemia). *Mol. Genet. Metab.*, **76**, 243–249.
22. Sakata, Y., Owada, Y., Sato, K., Kojima, K., Hisanaga, K., Shinka, T., Suzuki, Y., Aoki, Y., Satoh, J., Kondo, H. *et al.* (2001) Structure and expression of the glycine cleavage system in rat central nervous system. *Brain Res. Mol. Brain Res.*, **94**, 119–130.
23. Fleming, A. and Copp, A.J. (1998) Embryonic folate metabolism and mouse neural tube defects. *Science*, **280**, 2107–2109.

24. Applegarth, D.A., Toone, J.R. and Lowry, R.B. (2000) Incidence of inborn errors of metabolism in British Columbia, 1969–1996. *Pediatrics*, **105**, e10.
25. Nyhan, W.L. (1989) Nonketotic hyperglycinemia. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic Basis of Inherited Disease*. McGraw-Hill, Inc., New York, 743–753.
26. Kure, S., Kato, K., Dinopoulos, A., Gail, C., DeGrauw, T.J., Christodoulou, J., Bzduch, V., Kalmanchey, R., Fekete, G., Trojovsky, A. *et al.* (2006) Comprehensive mutation analysis of GLDC, AMT, and GCSH in nonketotic hyperglycinemia. *Hum. Mutat.*, **27**, 343–352.
27. Harris, M.J. (2008) Insights into prevention of human neural tube defects by folic acid arising from consideration of mouse mutants. *Birth Defects Res. A Clin. Mol. Teratol.*, **85**, 331–339.
28. Watanabe, M., Osada, J., Aratani, Y., Kluckman, K., Reddick, R., Malinow, M.R. and Maeda, N. (1995) Mice deficient in cystathionine β-synthase: animal models for mild and severe homocyst(e)inemia. *Proc. Natl Acad. Sci. USA*, **92**, 1585–1589.
29. Champion, K.M., Cook, R.J., Tollaksen, S.L. and Giometti, C.S. (1994) Identification of a heritable deficiency of the folate-dependent enzyme 10-formyltetrahydrofolate dehydrogenase in mice. *Proc. Natl Acad. Sci. USA*, **91**, 11338–11342.
30. Chen, Z., Karaplis, A.C., Ackerman, S.L., Pogribny, I.P., Melnyk, S., Lussier-Cacan, S., Chen, M.F., Pai, A., John, S.W., Smith, R.S. *et al.* (2001) Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. *Hum. Mol. Genet.*, **10**, 433–443.
31. MacFarlane, A.J., Liu, X., Perry, C.A., Flodby, P., Allen, R.H., Stabler, S.P. and Stover, P.J. (2008) Cytoplasmic serine hydroxymethyltransferase regulates the metabolic partitioning of methylenetetrahydrofolate but is not essential in mice. *J. Biol. Chem.*, **283**, 25846–25853.
32. Di, P.E., Sirois, J., Tremblay, M.L. and Mackenzie, R.E. (2002) Mitochondrial NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase is essential for embryonic development. *Mol. Cell. Biol.*, **22**, 4158–4166.
33. Swanson, D.A., Liu, M.L., Baker, P.J., Garrett, L., Stitzel, M., Wu, J.M., Harris, M., Banerjee, R., Shane, B. and Brody, L.C. (2001) Targeted disruption of the methionine synthase gene in mice. *Mol. Cell. Biol.*, **21**, 1058–1065.
34. Elmore, C.L., Wu, X., Leclerc, D., Watson, E.D., Bottiglieri, T., Krupenko, N.I., Krupenko, S.A., Cross, J.C., Rozen, R., Gravel, R.A. and Matthews, R.G. (2007) Metabolic derangement of methionine and folate metabolism in mice deficient in methionine synthase reductase. *Mol. Genet. Metab.*, **91**, 85–97.
35. Field, M.S., Anderson, D.D. and Stover, P.J. Mthfs is an essential gene in mice and a component of the purinosome. *Front. Genet.* http://www.frontiersin.org/nutrigenomics/10.3389/fgene.2011.00036/abstract.
36. Spiegelstein, O., Mitchell, L.E., Merriweather, M.Y., Wicker, N.J., Zhang, Q., Lammer, E.J. and Finnell, R.H. (2004) Embryonic development of folate binding protein-1 (Folbp1) knockout mice: effects of the chemical form, dose, and timing of maternal folate supplementation. *Dev. Dyn.*, **231**, 221–231.
37. Burren, K.A., Savery, D., Massa, V., Kok, R.M., Scott, J.M., Blom, H.J., Copp, A.J. and Greene, N.D.E. (2008) Gene-environment interactions in the causation of neural tube defects: folate deficiency increases susceptibility conferred by loss of *Pax3* function. *Hum. Mol. Genet.*, **17**, 3675–3685.
38. Beaudin, A.E., Abarinov, E.V., Noden, D.M., Perry, C.A., Chu, S., Stabler, S.P., Allen, R.H. and Stover, P.J. (2011) Shmt1 and de novo thymidylate biosynthesis underlie folate-responsive neural tube defects in mice. *Am. J. Clin. Nutr.*, **93**, 789–798.
39. Nijhout, H.F., Reed, M.C., Lam, S.L., Shane, B., Gregory, J.F. III and Ulrich, C.M. (2006) In silico experimentation with a model of hepatic mitochondrial folate metabolism. *Theor. Biol. Med. Model.*, **3**, 40.
40. Pike, S.T., Rajendra, R., Artzt, K. and Appling, D.R. (2010) Mitochondrial C1-tetrahydrofolate synthase (MTHFD1L) supports the flow of mitochondrial one-carbon units into the methyl cycle in embryos. *J. Biol. Chem.*, **285**, 4612–4620.
41. Dunlevy, L.P.E., Burren, K.A., Mills, K., Chitty, L.S., Copp, A.J. and Greene, N.D.E. (2006) Integrity of the methylation cycle is essential for mammalian neural tube closure. *Birth Defects Res. A*, **76**, 544–552.
42. Greene, N.D., Stanier, P. and Moore, G.E. (2011) The emerging role of epigenetic mechanisms in the aetiology of neural tube defects. *Epigenetics*, **6**, 875–893.
43. Apostolidou, S., Abu-Amero, S., O'Donoghue, K., Frost, J., Olafsdottir, O., Chavele, K.M., Whittaker, J.C., Loughna, P., Stanier, P. and Moore, G.E. (2007) Elevated placental expression of the imprinted PHLDA2 gene is associated with low birth weight. *J. Mol. Med.*, **85**, 379–387.
44. Niwa, H., Yamamura, K. and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*, **108**, 193–199.
45. Oda, M., Kure, S., Sugawara, T., Yamaguchi, S., Kojima, K., Shinka, T., Sato, K., Narisawa, A., Aoki, Y., Matsubara, Y. *et al.* (2007) Direct correlation between ischemic injury and extracellular glycine concentration in mice with genetically altered activities of the glycine cleavage multienzyme system. *Stroke*, **38**, 2157–2164.
46. Fujiwara, K., Okamura-Ikeda, K. and Motokawa, Y. (1991) Lipoylation of H-protein of the glycine cleavage system. The effect of site-directed mutagenesis of amino acid residues around the lipoyllysine residue on the lipoate attachment. *FEBS Lett.*, **293**, 115–118.
47. Wlodarczyk, B.J., Tang, L.S., Triplett, A., Aleman, F. and Finnell, R.H. (2006) Spontaneous neural tube defects in splotch mice supplemented with selected micronutrients. *Toxicol. Appl. Pharmacol.*, **213**, 55–63.
48. Essien, F.B. and Wannberg, S.L. (1993) Methionine but not folinic acid or vitamin B-12 alters the frequency of neural tube defects in *Axd* mutant mice. *J. Nutr.*, **123**, 27–34.
49. Nakai, T., Nakagawa, N., Maoka, N., Masui, R., Kuramitsu, S. and Kamiya, N. (2005) Structure of P-protein of the glycine cleavage system: implications for nonketotic hyperglycinemia. *EMBO J.*, **24**, 1523–1536.

Seizure 21 (2012) 55–60

Contents lists available at ScienceDirect

Seizure

journal homepage: www.elsevier.com/locate/yseiz

Case report

Epilepsy in RAS/MAPK syndrome: Two cases of cardio-facio-cutaneous syndrome with epileptic encephalopathy and a literature review

Masao Adachi [a,*], Yu Abe [b], Yoko Aoki [b], Yoichi Matsubara [b]

[a] Department of Pediatrics, Kakogawa Hospital Organization, Kakogawa West-City Hospital, 384-1 Hiratsu, Yoneda-cho, Kakogawa, Hyogo 675-8611, Japan
[b] Department of Medical Genetics, Tohoku University School of Medicine, Sendai, Japan

ARTICLE INFO

Article history:
Received 30 May 2011
Accepted 29 July 2011

Keywords:
Cardio-facio-cutaneous syndrome
RAS/MAPK pathway
Neurological impairment
Seizure

ABSTRACT

We report two individual cases of cardio-facio-cutaneous (CFC) syndrome with severe neurological impairment consisting of infantile spasms with hypsarrhythmia and refractory epilepsy with multifocal epileptic paroxysms such as modified hypsarrhythmia. Both cases shared diffuse brain atrophy and severely delayed myelination on neuroimaging. Genetic analysis revealed individual heterozygous mutations in the KRAS (phenotype of CFC/Noonan syndrome) and BRAF genes (phenotype of CFC syndrome). Neurological impairment in cases with mutations in the RAS/MAPK (mitogen activated protein kinase) signal pathway may be more severe, and could be linked to some forms of refractory epilepsy, especially epileptic encephalopathy that includes infantile spasms.

1. Introduction

Cardio-facio-cutaneous (CFC) syndrome is a very rare and sporadic disease that includes the characteristics of dysmorphic facial appearance, ectodermal abnormalities, cardiac abnormalities, growth retardation and neuro-developmental delay. This syndrome is categorized as one of the RAS/MAPK syndromes, which cause altered signal transduction of the RAS/MAPK (mitogen activated protein kinase) pathway, including BRAF, MEK1/2, and KRAS.[1–3] Compared with other RAS/MAPK syndromes, such as Costello syndrome and Noonan syndrome, CFC syndrome exhibits a more severe phenotype including severe neurological impairment, seizures, and developmental delay. We describe the clinical details of neurological findings in two cases of genetically determined CFC syndrome which displayed refractory epilepsies diagnosed as infantile spasms and other epileptic encephalopathy, and we then compare our results with those of similar literature findings.

2. Case reports

2.1. Case 1: six-year-old boy

A large-for-date boy was delivered as the first child to healthy and non-consanguineous Japanese parents (mother 42 years old and father 31 years old) at 32 weeks of gestational age with a birth weight of 3758 g and with moderate neonatal asphyxia (an Apgar score of 6 at 5 min after birth) following a normal pregnancy. The patient was intensively treated in the neonatal intensive care unit (NICU) in our hospital. Postnatal screening showed fetal hydrops with heart failure due to severe pulmonary valve stenosis which was treated with diuretics and beta blockers. Peculiar craniofacial features included "coarse face," curly hair, prominent forehead, downslanting palpebral fissures, short nose and broad nasal tip with anteverted nares, low-set dysmorphic and posteriorly angulated ears, abnormal skin (loose and pigmented skin with deep furrows and multiple lentigo, wrinkled palms with deep palmar and plantar creases), webbed neck, chest deformity, and micromelic dwarfism (Fig. 1A).

At three days postnatal, myoclonic seizures of the extremities occurred which were controlled by administration of bolus midazolam (MDL). At the age of 11 months, he developed repetitive series-formed tonic spasms, and the interictal electroencephalogram (EEG) showed hypsarrhythmia (Fig. 1C). Valproic acid (VPA), clonazepam (CZP), and zonisamide (ZNS) were ineffective in reducing seizure frequency and improving EEG findings, and complete remission was only obtained by one course of low dose (0.025 mg/kg) adrenocorticotropic hormone (ACTH). Since undergoing ACTH therapy, he has had no episodes of epileptic seizures while undergoing VPA monotherapy until the present age of six years, but his most recent (interictal) EEG showed asynchronous, high-voltage slow waves with irregular spike-waves, or polyspikes with/without waves dominantly in the right temporal-occipital region (Fig. 1D). Magnetic resonance imaging (MRI) at three years of age revealed agenesis of the corpus callosum, ventricular dilatation, diffuse cortical atrophy and severely delayed myelination (Fig. 1B). Tracheotomy was performed and persistent mechanical ventilation

* Corresponding author. Tel.: +81 79 432 3531; fax: +81 79 432 3672.
E-mail address: ama-p@rc4.so-net.ne.jp (M. Adachi).

1059-1311/$ – see front matter © 2011 British Epilepsy Association. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.seizure.2011.07.013

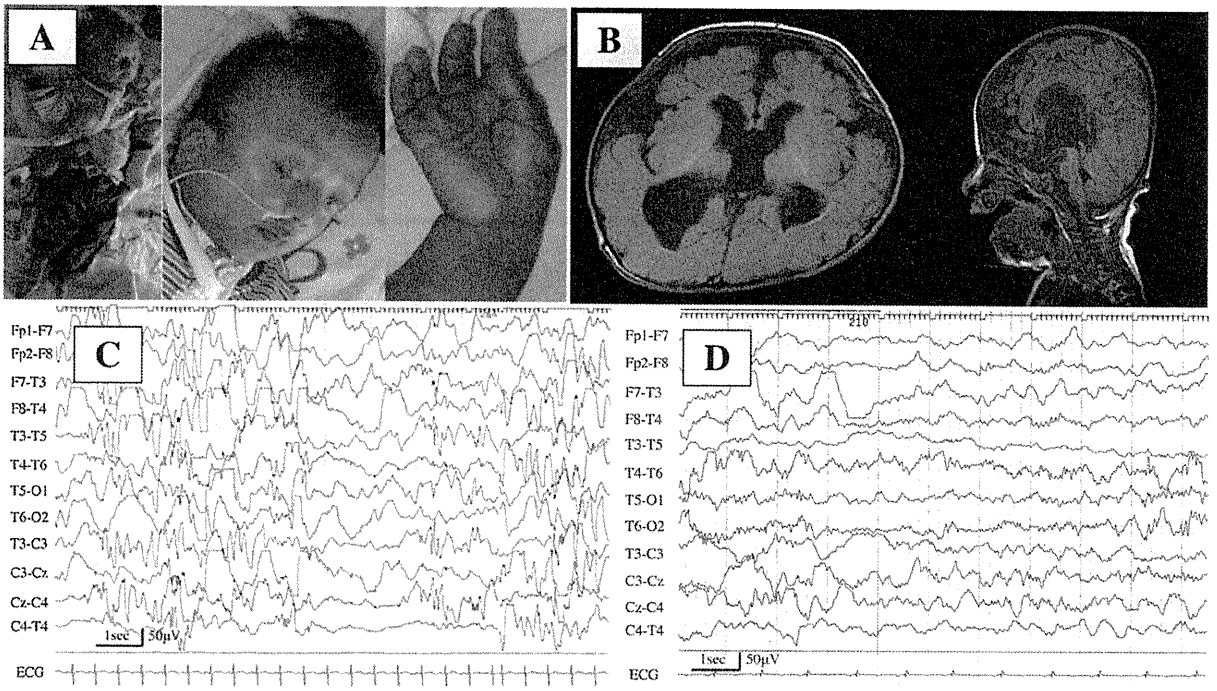

Fig. 1. Case 1. "Coarse face," curly hair, prominent forehead, dysmorphic ears, abnormal loose and pigmented skin, webbed neck, chest deformity, and micromelic dwarfism at six years (A), agenesis of the corpus callosum, ventricular dilatation, diffuse cortical atrophy and severely delayed myelination on MRI images at three years (B), hypsarrhythmia at 11 months (C), asynchronous, high-voltage slow waves with irregular spike-wave or polyspikes with/without waves dominantly in the right temporal-occipital region at six years of age (D) on EEG.

was initiated for severe dyspnea with laryngo/tracheomalacia. Percutaneous endoscopic gastrostomy (PEG) was performed for repetitive aspiration pneumonia caused by dysphagia. He has been diagnosed as mentally retarded and had not developed any expressive language. Additionally, he suffers from truncal hypotonia with increased muscle tone and joint contractures in his extremities. He has been profoundly delayed in terms of physical and mental development due to his severe motor and intellectual disabilities.

His karyotype was 46,XY and genetic screening confirmed a heterozygous nucleotide change within exon 5 of the KRAS gene (c.458A > T), causing the amino acid substitution D153V, whose phenotype was CFC/Noonan syndrome.

2.2. Case 2: four-year-old girl

An appropriate-for-date girl (35 weeks of gestational age with a birth weight of 2624 g) without asphxial episodes was born to healthy and non-consanguineous parents (mother 30 years old and father 35 years old) who had previously given birth to three healthy baby girls. Following delivery, several surface anomalies were noted, such as an odd-looking "coarse" face (prominent forehead, short nose and broad nasal bridge with anteverted nares, downslanting palpebral fissures, and low-set dysmorphic ears), curly and sparse hair, abnormal skin manifestations (loose, pigmented skin with multiple lentigo, wrinkled palms with deep palmar and plantar creases), narrow chest, and hypotonic micromelic dwarfism (Fig. 2A). Postnatal screening revealed cardiac failure due to severe hypertrophic cardiomyopathy, resulting in chronic heart failure, which necessitated the administration of diuretics and beta blockers.

On admission to our NICU, a subtle seizure occurred and was only controlled following the infusion of phenobarbital (PB) and MDL. After this episode, seizures have been severely refractory and uncontrolled despite the use of a majority of antiepileptic drugs, including PB, VPA, CZP, ZNS, carbamazepine, phenytoin, primidone, nitrazepam, clobazam, topiramate, lamotirigine, gabapentin, levetiracetam. Seizures are composed of repetitive brief tonic spasms, tonic-clonic (sometimes developing to status epileptics), myoclonic, and complex partial seizures (sometimes evolving to generalized tonic-clonic seizure [GTCS]), all occurring daily and frequently. Interictal EEG revealed modified hypsarrhythmia at one year of age (Fig. 2C), and her most recent (interictal) EEG showed continuous high-voltage spike or polyspikes with/without slow waves mainly in the left centro-temporal-parietal region at four years of age (Fig. 2D). ACTH therapy has not been introduced because of moderate cortical atrophy with delayed myelination and hypoplastic corpus callosum on cranial MRI images noted at two years of age (Fig. 2B). In addition to seizures, she has exhibited frequent involuntary movement, consisting of dystonia, athetosis, and myoclonus, all resistant to various muscle-relaxant drugs.

She had frequently developed episodes of dyspnea due to congenital laryngo/tracheomalacia, which resulted in tracheotomy and persistent mechanical ventilation during night sleep before two years of age, but recurrent aspiration pneumonia caused by dysphagia finally required PEG. She has been profoundly mentally retarded and unable to speak any words. She has been unable to sit unassisted because of general hypotonia and joint contractures in her extremities. Overall, she has exhibited severe motor and intellectual disabilities.

Her karyotype was 46,XX and advanced genetic screening confirmed a heterozygous nucleotide change within exon 12 of the BRAF gene (c.1454T > C), causing the amino acid substitution L485S, whose clinical phenotype was CFC syndrome.

3. Discussion

The different types of RAS/MAPK syndrome have many overlapping characteristics, including craniofacial manifestations, cardiac malformations, cutaneous, musculoskeletal, gastrointestinal, ocular abnormalities, and neuro-cognitive impairment,

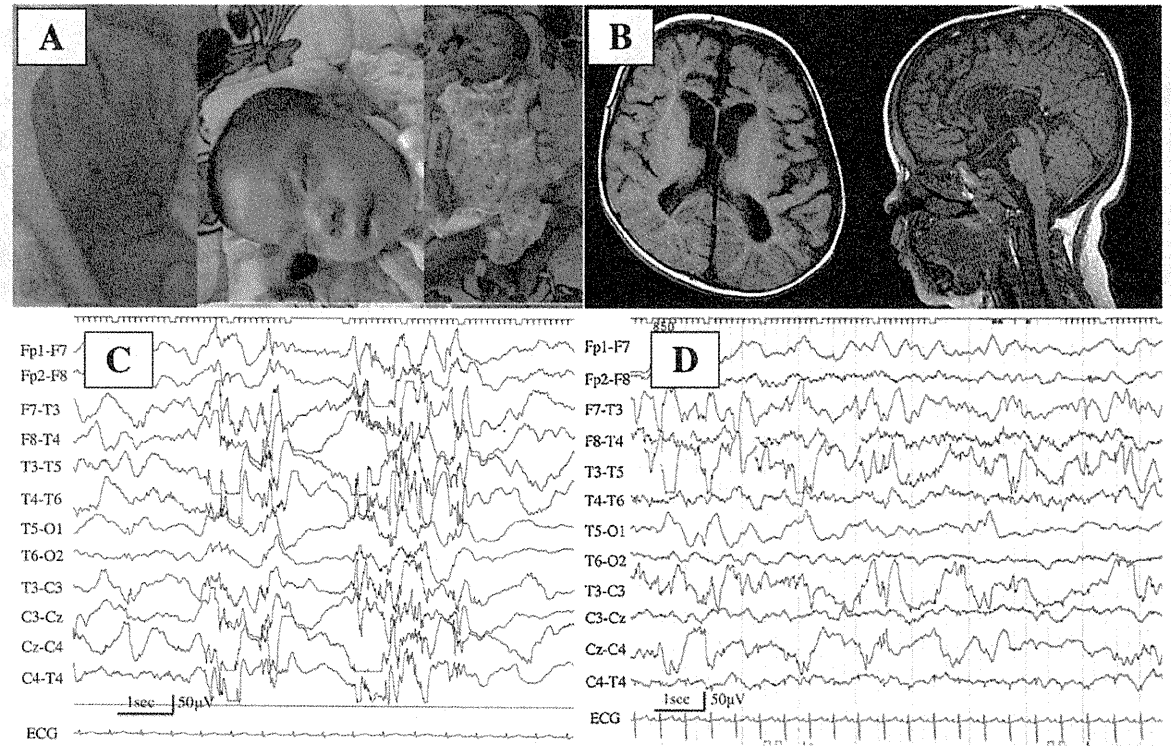

Fig. 2. Case 2. Odd looking "coarse" face (prominent forehead, short nose, and low-set dysmorphic ears, curly sparse hair), abnormal loose, pigmented skin with multiple lentigo, narrow chest, and hypotonic micromelic dwarfism at four years (A), diffuse brain atrophy with delayed myelination and hypoplastic corpus callosum on MRI images at two years (B), modified hypsarrhythmia at one year (C), and continous high-voltage spike or polyspikes with/without slow waves mainly in the left centrotemporalparietal region at four years of age (D) on EEG.

including hypotonia and seizures, caused by dysregulation of signaling in the RAS/MAPK pathway due to mutations mainly in BRAF, MEK1, or MEK2.[1–3] Correlation between confirmed mutations and non-neurological, cardiovascular, cutaneous, and musculoskeletal abnormalities in CFC patients have been discussed,[1–3] but detailed analyses of their associated neurological impairments, especially epileptic conditions, have been sorely lacking.

Only a few previous reports[4–10] of CFC syndrome mentioned associated neurological impairment, especially seizures and EEG findings, but were lacking in terms of their detailed clinical features and courses. Gross-Tsur et al. described the neurological status of 16 patients with CFC syndrome (genetically undetermined cases) in their report with a literature review,[4] including six EEG findings (generalized dysrhythmia [grade, and ungraded], low voltage, focal activity, and episodes of spike and slow 2 Hz with slow background activity). Recently, Yoon et al. mentioned the seizure types and EEG findings in 12 of 15 cases, including four cases of infantile spasms with hypsarrhythmia on EEG.[7] Moreover, Aizaki et al. reported a case of CFC syndrome with infantile spasms, suggesting that seizures with CFC syndrome were refractory despite the administration of various types of anticonvulsants and that the neuro-developmental delay caused by CFC syndrome is severe.[10]

Both cases in the present report exhibited infantile spasms with severely abnormal EEG (modified hypsarrhythmia). Case 1 has been remitted with ACTH therapy and Case 2 remains uncontrolled despite the administration of various types of anticonvulsants. Upon reviewing previous literature[4–10] describing the epileptic conditions and neuroimages in patients with cardio-facio-cutaneous syndrome (Table 1), 62 cases were discovered which mentioned their epileptic condition which included 12 cases (19.3%) with infantile spasms or brief tonic spasms, each of which were accompanied by hypsarrhythmia or modified hypsarrhythmia on EEG. Other cases also develop various types of seizures, GTCS (sometimes evolving to status epileptics), and complex partial seizure, with severe abnormal EEG, consisting of generalized or partial epileptiform activities. Most of the seizures in these patients still remain uncontrolled despite the use of various types of anticonvulsants. Based on the two present cases and the literature review, the high complication rate of infantile spasms in CFC syndrome suggests that there may be specific factors relating to refractory epilepsy, especially epileptic encephalopathy, in the RAS/MAPK signaling pathway.

As for correlations between genotype and epileptic phenotype, D153V mutation in the KRAS gene (as seen in Case 1) was previously reported[11–13] in six cases (two of CFC syndrome, three of Noonan syndrome, and one of CFC/Noonan syndrome), but in none of these cases did the patient develop seizures. Accordingly, this mutation may be unrelated to epileptic severity. On the other hand, it is noteworthy that the mutation L485S in the BRAF gene (as seen in Case 2) has been recently reported to be detected in a patient of CFC syndrome with infantile spasms following a refractory therapeutic course.[10] This point mutation may be related to the severity of epileptic conditions in RAS/MAPK syndrome. In addition, this report[10] described the efficacy of a ketogenic diet (KD) to reduce seizure frequency, but in the present Case 2 patient with the same mutation in the BRAF gene, KD has not been introduced because of severe thinness despite adequate tube nutrition.

In a recent report of CFC patients, neuroimaging played an important role in the diagnosis of this syndrome.[14] Most of the 62 patients with CFC syndrome in the present review shared severe abnormal neuroimaging, including hydrocephalus, agenesis/hypoplasia of the corpus callosum, ventricular dilatation, cortical

Table 1
Epileptic conditions and neuroimages in patients with cardio-facio-cutaneous syndrome: present cases and those from a literature review.

Ref.	Gene	Mutation	Sex	Age of seizure onset	Seizure type	Interictal EEG findings	Anticonvulsant therpay	Seizure prognosis	Neurodevelopmental delay	Neuroimaging (brain MRI)
Gross-Tsur et al.[4] ($n=1$)	N.A.	N.A.	M	1 y 9 mo	Lennox–Gastaut syndrome	Multiple episodes of spike and slow wave activity, 2 Hz. The background activity was abnormally slow.	VPA, CZP	Decrease in seizures	Hypotonia, ataxia, lack of language skill, extreme hyperactivity	Normal
Raymond and Holmes[5] ($n=2$)	N.A.	N.A.	F	–	No seizure	Decrease in anterior voltages, no epileptiform activity (postnatal screening)	–	–	No motor delay, marked language delay	External hydrocephalus with widened subarachnoid space, cortical atrophy in the frontal and temporal lobes (CT)
	N.A.	N.A.	M	–	No seizure	N.A.	–	–	No motor delay, marked language delay	Marked cortical atrophy (CT)
Sabatino et al.[6] ($n=2$)	N.A.	N.A.	M	1 y 3 mo	Tonic-clonic (SE)	Focal activity in the bilateral posterior areas	N.A.	N.A.	Moderate to severe	Cortical atrophy, ventriculomegaly
	N.A.	N.A.	F	6 y	GTCS	Irritative waves and generalized disorganization, frequent focal spikes in the right regions, sometimes in contralateral areas.	PB	Controlled	Moderate to severe	Diffuse cortical atrophy
Yoon et al.[7] ($n=15$)	MEK1	F53S	F	15 y 10 mo	GTCS, Abs, CPS	Generalized spikes/slow waves ($n=5$), hypsarrhythmia ($n=4$), focal epileptiform discharges ($n=3$)	ZNS, LEV	Not described in detail. Polytherapy required in 9 of 15 cases, suggesting that seizure control is often difficult.	Severe	Ventriculomegaly and hydrocephalus (66%), prominent Vircho- Robin spaces (20%), cortical atrophy, prominence of CFS spaces wih macrocephaly, benign extraventricular obstructive hydrocephalus. (some cases)
	BRAF	L485F	M	2 wk	CPS, sGTCS, Abs		OXC, DZP,		Severe	
	BRAF	F468S	F	11 y	GTCS		CBZ, PB		Profound	
	BRAF	Q257R	M	2 y 6 mo	Abs, focal		VPA		Mild	
	BRAF	del E11	F	1 y 6 mo	IS		TPM, CZP, VPA, PSL		Severe	
	BRAF	Q257R	M	3 y	Not specified		CBZ		Severe	
	BRAF	F595L	F	6 mo	IS, vocal motor, CPS		Felbamate, ZNS, CZP		Profound	
	BRAF	T599R	F	3 y	Not specified		OXC		Profound	
	BRAF	G534R	M	5 y	GTCS, Abs		OXC		Profound	
	BRAF	L485S	M	4 mo	GTCS, CPS, IS		TPM, CZP, VPA, DZP		N.A.	
	MEK1	Y130C	F	2y	Not specified		LEV		N.A.	
	BRAF	D638E	F	1 y 6 mo	GTCS, Abs		LEV, PHT		Profound	
	BRAF	K499N	F	7 mo	GTCS, Abs		LTG, CBZ, CZP		Severe	
	MEK1	Y130N	F	1 y	CPS		OXC		Profound	
	MEK1	G128V	F	5 mo	IS		PB, LTG, VPA, CZP		N.A.	

Table 1 (*Continued*)

Ref.	Gene	Mutation	Sex	Age of seizure onset	Seizure type	Interictal EEG findings	Anticonvulsant therpay	Seizure prognosis	Neurodevelopmental delay	Neuroimaging (brain MRI)
Armour and Allanson[8] ($n=38$)	BRAF (15/32 cases) MEK1(2/4 cases) MEK2(1/2 cases)	N.A.	N.A.	IS ($n=5$), Abs ($n=4$), GTCS ($n=4$), CPS ($n=4$)	N.A.	N.A.	Respondents 49%	All significant delay (available in 27cases)	Hydrocephaly (2), ventriculomegaly (9), reduced white matter (6), thin corpus callosum (3), cerebral atrophy (3), delayed myelination (3), Chiari 1 malformation (1), pachygyria (1), nodulat heterotopia (1), abnormal migration (1), cerebellar calcification (1) available on 23 cases)	
Demir et al.[9] ($n=1$)	BRAF	F468S	F	N.A.	Recurrent clonic seizures	Epileptiform discharges in the right front central temporal region	VPA, CBZ, TPM	Controlled	Mental/motor/ language delay	Mild frontoparietal cortical atrophy, mildly dilated ventricles, thinning of the posterior part of the corpus callosum
Aizaki et al.[10] ($n=1$)	BRAF	L485S	F	2 mo	Brief tonic spasms (repetitive)	Asynchronous, high-voltage slow waves with multifocal sharp waes appeared with bilateral pariet-occipital predominance	VPA, VitB6, ZNS, CLB, PB, ACTH, KD, Clorazepate dipotassium	Uncontrolled	Profound	Hypoplastic corpus callosum, moderate brain atrophy, delayed myelination, ambiguous coorticomedullary boundary in the right posterior temporal lobe
Present cases ($n=2$)	KRAS	D153V	M (Case 1)	3 mo/11 mo	Myo/IS	Hypsarrhythmia (at 11 mos), asynchronous, high-voltage slow waves with irregular spike-wave, or polyspikes with/without waves dominantly in the right temporal-occipital region (at 6 yrs)	MDL/VPA, CZP, ZNS, ACTH	Controlled	Profound	Diffuse cortical atriphy, ventricular dulatation, agenesis of the corpus callosum, delayed myelination
	BRAF	L485S	F (Case 2)	Day 0	Subtle, brief tonic spasms, CPS, GTCS	Modified hypsarrhythmia (at 1 yr), Continous high-voltage spike or polyspikes with/without slow waves in the left centro-temporalparietal region (at 4 yrs)	MDL, VPA, CZP, NZP, PB, CBZ, ZNS, CLB, PHT, PRM, GAP, TPM, LTG, LEV, TRH, ST	Uncontrolled	Profound	Diffuse cortical atrophy, ventricular dulatation, hypoplastic corpus callosum, delayed myelination

GTCS: generalized tonic-clonic seizure, sGTCS: secondarily generalized tonic-clonic seizure, CPS: complex partial seizure, Abs: absence seizure, Myo: myoclonic seizure, IS: infantile spasms, SE: status epileptics, Subtle: subtle seizure.

PB: phenobarbital, VPA: valproic acid, CBZ: carbamazepine, ZNS: zonisamide, PHT:pheytoin, PRM: primidon, CZP: clonazepam, CLB: clobazam, NZP: nitrazepam, DZP: diazepam, MDL: midazolam, ST: sultiame, VitB6: vitamin B6, GAP: gabapentin, TPM: topiramate, LTG: lamotrigine, LEV: levetiracetam, OXC: oxcarbazepine, ACTH: adrenocorticotropic hormone, PSL: prednisone, KD: ketogenic diet, CSF: cerebrospinal fluid, MRI: magnetic resonance image, CT: computed tomography, SE: status epilepticus, N.A.: not applicable.

atrophy, and delayed myeliation, resulting in neuro-developmental delay ranging from 'moderate to severe' to 'profound', all of which distinguish CFC syndrome from the other types of RAS/MAPK syndrome (Noonan and Costello syndromes).

More cases will need to be studied in order to clarify the genotype–phenotype correlations of several genes in the RAS/MAPK signaling pathway associated with refractory epilepsy.

Conflict of interest

The authors report no conflict of interest.

Acknowledgements

We thank the two patients and their families for their willingness to be included in this report.

References

1. Aoki Y, Niihori T, Narumi Y, Kure S, Matsubara Y. The RAS/MAPK syndromes: novel roles of the RAS pathway in human genetic disorders. *Hum Mutat* 2008;**29**:992–1006.
2. Rauen KA, Schoyer L, McCormick F, Lin AE, Allanson JE, Stevenson DA, et al. Proceedings from the 2009 genetic syndromes of the Ras/MAPK pathway: from bedside to bench and back. *Am J Med Genet A* 2010;**152A**:4–24.
3. Rauen KA, Banerjee A, Bishop WR, Lauchle JO, McCormick F, McMahon M, et al. Costello and cardio-facio-cutaneous syndromes: moving toward clinical trials in RASopathies. *Am J Med Genet C Semin Med Genet* 2011;**157**(2):136–46.
4. Gross-Tsur V, Gross-Kieselstein E, Amir N. Cardio-facio cutaneous syndrome: neurological manifestations. *Clin Genet* 1990;**38**:382–6.
5. Raymond G, Holmes LB. Cardio-facio-cutaneous (CFC) syndrome: neurological features in two children. *Dev Med Child Neurol* 1993;**35**:727–32.
6. Sabatino G, Verrotti A, Domizio S, Angeiozzi B, Chiarelli F, Neri G. The cardio-facio-cutaneous syndrome: a long-term follow-up of two patients, with special reference to the neurological features. *Childs Nerv Syst* 1997;**13**:238–41.
7. Yoon G, Rosenberg J, Blaser S, Rauen KA. Neurological complications of cardio-facio-cutaneous syndrome. *Dev Med Child Neurol* 2007;**49**:894–9.
8. Armour CM, Allanson JE. Further delineation of cardio-facio-cutaneous syndrome: clinical features of 38 individuals with proven mutations. *J Med Genet* 2008;**45**:249–54.
9. Demir E, Mancano G, Pomponi MG, Ozcelik A, Gucuyener K, Neri G. Cardio-facio-cutaneous syndrome: phenotypic variability and differential diagnosis in 3 cases with de novo BRAF mutations. *Neuropediatrics* 2010;**41**:127–31.
10. Aizaki K, Sugai K, Saito Y, Nakagawa E, Sasaki M, Aoki Y, et al. Cardio-facio-cutaneous syndrome with infantile spasms and delayed myelination. *Brain Dev* 2011;**33**:166–9.
11. Niihori T, Aoki Y, Narumi Y, Neri G, Cavé H, Verloes A, et al. Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. *Nat Genet* 2006;**38**:294–6.
12. Schubbert S, Zenker M, Rowe SL, Böll S, Klein C, Bollag G, et al. Germline KRAS mutations cause Noonan syndrome. *Nat Genet* 2006;**38**:331–6.
13. Carta C, Pantaleoni F, Bocchinfuso G, Stella L, Vasta I, Sarkozy A, et al. Germline missense mutations affecting KRAS Isoform B are associated with a severe Noonan syndrome phenotype. *Am J Hum Genet* 2006;**79**:129–35.
14. Papadopoulou E, Sifakis S, Sol-Church K, Klein-Zighelboim E, Stabley DL, Raissaki M, et al. CNS imaging is a key diagnostic tool in the evaluation of patients with CFC syndrome: two cases and literature review. *Am J Med Genet A* 2011;**155**:605–11.

LINK TO ORIGINAL ARTICLE
LINK TO RESPONSE

Mutation (variation) databases and registries: a rationale for coordination of efforts

Arleen D. Auerbach, John Burn, Jean-Jacques Cassiman, Mireille Claustres, Richard G. H. Cotton, Garry Cutting, Johan T. den Dunnen, Mona El-Ruby, Aida Falcon Vargas, Marc S. Greenblatt, Finlay Macrae, Yoichi Matsubara, David L. Rimoin, Mauno Vihinen and Christine Van Broeckhoven

The importance of gene- or locus-specific databases (LSDBs) has recently been extolled in this journal (The case for locus-specific databases. *Nature Reviews Genetics* **12**, 378–379 (2011))[1]. Here we argue that coordination of international efforts for developing comprehensive mutation databases and patient phenotype registries is essential for optimal genetic health care.

Well-funded international efforts for setting up mutation databases or registries are crucial for several reasons. Many variants that are found during clinical testing worldwide are not submitted to databases, where they could form an important resource for patient care. Many laboratories and clinicians do not have the capacity or incentive to submit data to databases. This is especially the case in developing countries owing mainly to technical insufficiency, lack of public awareness, lack of international communications, the absence of the concept of DNA biobanking, national authority restrictions and lack of translation from original languages to English.

The Human Variome Project (HVP) was initiated to facilitate the collection of all variants in all genes from all countries and to include annotation of these variants for pathogenicity and relevance to clinical medicine[2]. It was established at a meeting in 2006 that was attended by representatives of the World Health Organization (WHO), the United Nations Educational, Scientific and Cultural Organization (UNESCO), the Organisation for Economic Co-operation and Development (OECD), the European Commission, March of Dimes, the US National Center for Biotechnology Information, the European Bioinformatics Institute (EBI) and 30 countries[3]. The third HVP meeting at UNESCO Headquarters in 2010 allowed the election of an International Scientific Advisory Committee and affirmation of a Roadmap[4]. Most recently, China has committed $300 million to the project[5], and UNESCO has awarded the HVP the status of 'NGO in operational relations with UNESCO'. Many working groups are establishing standards for collecting, presenting and sharing variation information.

Registries for inherited diseases have been developed in some countries, especially where therapies are available (for example, see REF. 6). Recently, there has been a call for global registries of rare diseases (more than 80% of which are genetic)[7,8]. Most recently, the US National Institutes of Health and the European Commission have developed the International Rare Diseases Research Consortium (IRDiRC)[9].

These two initiatives, the HVP and IRDiRC, have been developing essentially independently and in parallel. The HVP was driven by clinicians and laboratories wishing to have access to complete disease-associated variation information to support diagnostic advice and to facilitate the publication of novel mutations of interest. Recently, the focus has moved to collecting all mutations in all genes from all countries[10] as a means of assisting the interpretation of functional effects of genetic variations. The IRDiRC has been driven by patient groups who are anxious to achieve therapy for their families' diseases and to recruit cohorts for clinical trials in registries.

Practically, the promised funds from China in support of the HVP will allow 5,000 databases to be properly set up. If the decision is to set up these databases as both mutation and patient registries, this will assist both initiatives and avoid duplication.

Each group has their own networks, methodology, experts, data content and specifications. It would seem wasteful if two parallel systems were developed when many data are in common and when global reach is needed by both. In the case of the HVP, key components that are in place are a federated model, forums for sharing experiences, development of best informatics practices that are relevant to the task, and leadership. Clearly in the case of the IRDiRC, the key components are model registries, Orphanet experience and Genetic Alliance experience.

Future generations will pay the price for a failure to establish a joint international approach to the recording of and provision of access to human molecular variation, as such access is the most important step in approaching the diagnosis, and thus prevention, of inherited disorders.

The authors are all members of the International Scientific Advisory Committee of the Human Variome Project.

Arleen D. Auerbach is at The Rockefeller University, New York, New York 10065, USA.

John Burn is at the Institute of Genetic Medicine, Newcastle University, International Centre for Life, Newcastle upon Tyne NE1 3BZ, UK.

Jean-Jacques Cassiman is at the Center for Human Genetics, Forensic Medicine Campus, Katholieke Universiteit Leuven, Leuven, Belgium.

Mireille Claustres is at the Institut of Universitaire Clinical Research, University Hospital of Montpellier, Montpellier 34093, France.

Richard G. H. Cotton is at the Human Variome Project, University of Melbourne, Melbourne, Australia.

Garry Cutting is at the Johns Hopkins School of Medicine, Baltimore, Maryland, USA.

Johan T. den Dunnen is at Leiden University Medical Center, 2333 ZA Leiden, The Netherlands.

Mona El-Ruby is at the National Research Centre, Dokki, Cairo 12311, Egypt.

Aida Falcon Vargas is at the Centro Clinico Profesional Caracas, Anexo Hospital de Clinicas Caracas, San Bernardino, Caracas, Venezuela.

Marc Greenblatt is at the University of Vermont, Burlington, Vermont 05405, USA

Finlay Macrae is at The Royal Melbourne Hospital, Parkville 3050, Melbourne, Victoria, Australia.

Yoichi Matsubara is at Tohoku University School of Medicine, Sendai, Miyagi 980–8574, Japan.

David Rimoin is at the Cedars–Sinai Medical Center, Los Angeles, California 90048, USA.

Mauno Vihinen is at the University of Tampere and BioMediTech, Tampere, Finland

Christine Van Broeckhoven is at the VIB Department of Molecular Genetics, University of Antwerp, 2610 Antwerp, Belgium.

Correspondence to A.D.A.
e-mail: auerbac@mail.rockefeller.edu

doi:10.1038/nrg3011-c1
Published online 25 October 2011

1. Samuels, M. E. & Rouleau, G. A. The case for locus-specific databases. *Nature Rev. Genet.* **12**, 378–379 (2011).
2. Anonymous. What is the Human Variome Project? *Nature Genet.* **39**, 423 (2007).
3. Cotton, R. G. *et al.* Recommendations of the 2006 Human Variome Project meeting. *Nature Genet.* **39**, 433–436 (2007).
4. Kohonen-Corish, M. R. J. *et al.* How to catch all those mutations — the report of the third Human Variome Project Meeting, UNESCO Paris, May 2010. *Hum. Mutat.* **31**, 1374–1381 (2010).