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).

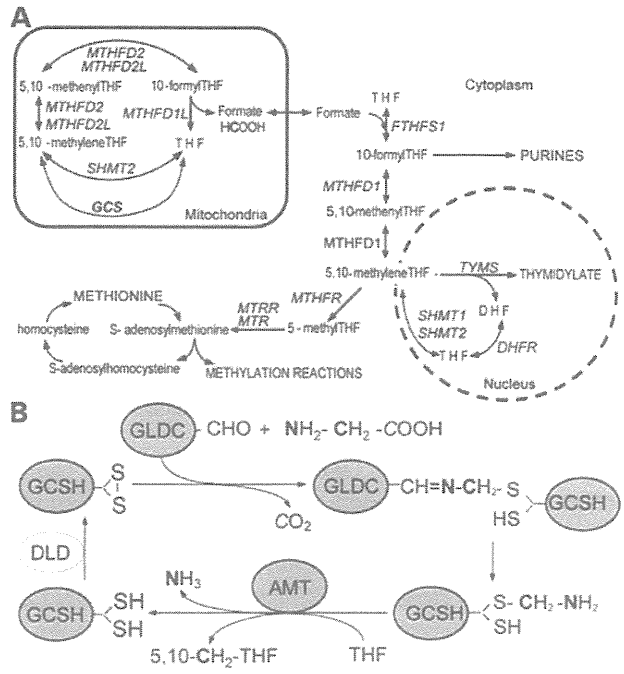

Figure 1. Schematic diagrams summarizing the key reactions of folate-mediated one-carbon metabolism and the GCS. (A) Folates donate and accept one-carbon units in the synthesis of purines, thymidylate and methionine. Mitochondrial FOCM supplies one-carbon units to the cytoplasm via formate. The GCS is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

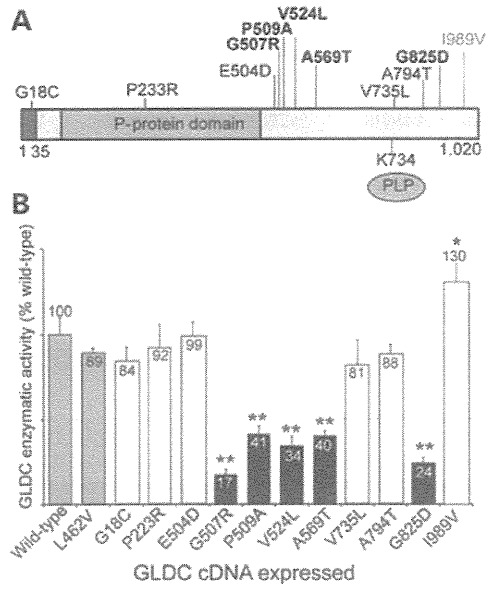

Figure 2. Characterization of *GLDC* missense mutations identified through DNA sequence analysis. (**A**) The schematic represents the 1020 amino acid residue GLDC polypeptide with the positions of the identified missense variants indicated. Mutations conferring significantly reduced activity (**B**) 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).

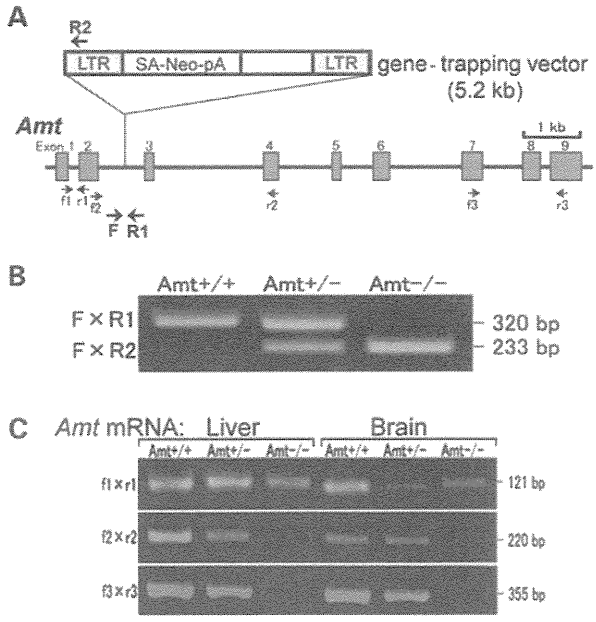

Figure 3. Generation of *Amt* knockout mouse by gene trapping. (A) The location of the gene-trap vector in *Amt* intron 2 in the ES cell line OST181110 was determined by inverse PCR. Mice carrying this mutation were generated using standard methods of blastocyst microinjection with OST181110 ES cells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

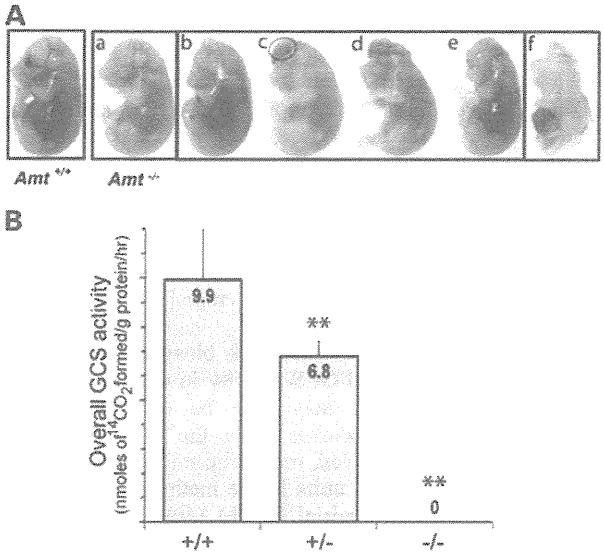

Figure 4. Mice lacking GCS activity exhibit NTDs. (A) Phenotypes of *Amt* mutant mice. NTDs were evident in the majority (88%) of $Amt^{-/-}$ fetuses (examples shown are at E17.5). Various types of NTDs were observed in $Amt^{-/-}$ fetuses, which principally affected the cranial region; a, no NTDs; b,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

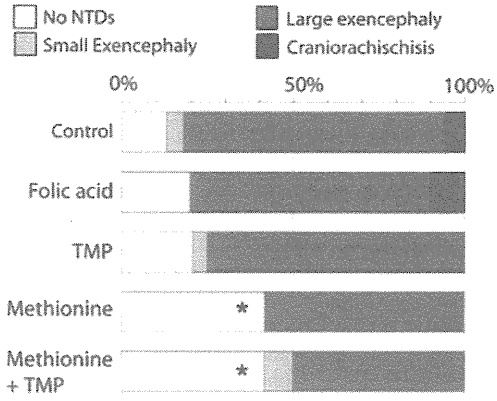

Figure 5. Maternal supplementation of *Amt* mutant embryos with folic acid, TMP or methionine. Maternal treatment with folic acid ($n = 10$ homozygous mutant fetuses) or TMP ($n = 12$) had no significant effect on NTD frequency, whereas the frequency of unaffected embryos was significantly increased 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.

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LINK TO ORIGINAL ARTICLE
LINK TO RESPONSE

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

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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.

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Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
The Human Genome Variation Society: www.hgvs.org
The Human Variome Project: www.humanvariomeproject.org
ALL LINKS ARE ACTIVE IN THE ONLINE PDF

CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Implantable Cardioverter Defibrillator for Progressive Hypertrophic Cardiomyopathy in a Patient With LEOPARD Syndrome and a Novel *PTPN11* Mutation Gln510His

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LEOPARD syndrome (LS), generally caused by heterozygous mutations in the *PTPN11* gene, is a rare autosomal-dominant multiple congenital anomaly condition, characterized by skin, facial, and cardiac abnormalities. Prognosis appears to be related to the type of structural, myocardial, and arrhythmogenic cardiac disease, especially hypertrophic cardiomyopathy (HCM). We report on a woman with LS and a novel Gln510His mutation in *PTPN11*, who had progressive HCM with congestive heart failure and nonsustained ventricular tachycardia, successfully treated with implantable cardioverter defibrillator (ICD). Comparing our patient to the literature suggests that specific mutations at codon 510 in *PTPN11* (Gln510Glu, Gln510His, but not Gln510Pro) might be a predictor of fatal cardiac events in LS. Molecular risk stratification and careful evaluations for an indication of ICD implantation are likely to be beneficial in managing patients with LS and HCM. © 2011 Wiley-Liss, Inc.

Key words: LEOPARD syndrome; *PTPN11*; codon 510; hypertrophic cardiomyopathy; nonsustained ventricular tachycardia; implantable cardioverter defibrillator

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INTRODUCTION

LEOPARD syndrome (LS) (OMIM#151100) is a rare autosomal-dominant multiple congenital anomaly condition, characterized by multiple lentigines, electrocardiographic (ECG) abnormalities, ocular hypertelorism, pulmonary stenosis, genital abnormalities, growth retardation, and sensorineural deafness [Sarkozy et al., 2008]. LS is caused by heterozygous missense mutations in the protein tyrosine phosphates, non-receptor type 11 gene (*PTPN11*) in roughly 85% of the cases [Digilio et al., 2002; Sarkozy et al., 2008]. The protein encoded by *PTPN11* functions as a cytoplasmic signaling transducer downstream of multiple receptors for growth factors, cytokines, and hormones, with a particular role through the RAS/mitogen activated protein kinase (MAPK) pathway [Sarkozy et al., 2008]. Disorders caused by mutations in various RAS/MAPK pathway components have recently been coined as "RASopathies", including Noonan syndrome, neurofibromatosis 1, cardio-facio-cutaneous syndrome, Costello syndrome, and LS [Rauen et al., 2010; Marin et al., 2011].

The prognosis of LS depends on the type of cardiovascular abnormality, especially hypertrophic cardiomyopathy (HCM) [Limongelli et al., 2008; Lehmann et al., 2009], however there have been few guidelines to manage complications. We report on a woman with LS and a novel Gln510His mutation in *PTPN11*, who had progressive HCM with congestive heart failure and nonsustained ventricular tachycardia, successfully treated with implantable cardioverter defibrillator (ICD) as for primary prevention of sudden death.

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CLINICAL REPORT

The proposita is a 38-year-old Japanese woman who underwent intracardiac repair of an atrial septal defect and pulmonary stenosis at age 2 years, when cardiac hypertrophy was detected. In childhood, she was easily exhausted after exercise and had growth retardation. At age 8 years, she was diagnosed with HCM with heart failure, though detailed laboratory data was not available. Oral administration of disopyramide and atenolol was initiated. In her 30s, she had generalized edema. Her plasma brain natriuretic peptide level was elevated at around 2,000 pg/ml (normal values, <18 pg/ml).

At age 37 years, she showed dyspnea, and was referred to our hospital. Her height was 143 cm (−3.0 SD) and weight was 40.8 kg (−1.6 SD). Her craniofacial features included hypertelorism, prominent eyes, a flat nose with anteverted nostrils, low-set posteriorly rotated ears, a long philtrum, thick lips, a high palate, and multiple caries (Fig. 1A). Her skeletal features included a short neck and short fingers with mild flexion contractures at the distal interphalangeal joints. She had numerous lentigines (congenital freckles) on the face (Fig. 1A) and café-au-lait spots on the back (Fig. 1B). She had no apparent hearing impairment. Her blood pressure was 130/66 mmHg, heart rate was 80 beats per minute, and SpO2 was 97% under administration of 1 L/min oxygen. Grade 2 systolic murmurs were heard at the 4th left intercostal space. The plasma brain natriuretic peptide level was 3,450 pg/ml. A chest radiograph showed cardiomegaly with a cardiothoracic ratio as 62% and pulmonary congestion. An ECG showed complete right bundle branch block and left axis deviation. Echocardiograph showed thickening of the interventricular septum as 23 mm (normal values, 7–12) and of the posterior wall of the left ventricle as 30 mm (normal values, 7–12), and tricuspid valve regurgitation with a pressure gradient as 35 mmHg. Pressure gradient of the left ventricular outlet was 21 mmHg at rest (stress echo was not performed to look for a provocable pressure gradient). Left ventricular end-diastolic volume was 20 ml (normal values, 56–136) and ejection fraction was 75% (normal values, >55). These findings were consistent with non-obstructive HCM with left ventricular hypertrophy and without low ejection fraction. The patient was treated with candesartan (angiotensin II receptor blocker), torasemide (diuretics), carvedilol (beta blocker), and amiodarone (antiarrhythmic agent), and her symptoms were improved with a decreased brain natriuretic peptide level to 1,720 pg/ml.

A delayed enhanced cardiac magnetic resonance imaging revealed severe concentric left ventricular hypertrophy with narrowing of the internal cavity and scattered hyper-enhancement regions that were suggested to be fibrosed myocardium [Moon et al., 2003]. A 24-hour Holter ECG showed 1,406 multifocal premature ventricular contractions and eight series of multifocal nonsustained ventricular tachycardia. An electrophysiological study, through a cardiac catheterization, demonstrated that polymorphic ventricular tachycardia was induced by programmed extrastimuli from the right ventricular apex with 400–250–240–230 ms, resulting in consciousness loss. According to the American College of Cardiology/the American Heart Association/the Heart Rhythm Society guidelines for device-based therapy [Epstein et al., 2008], she has two major risk factors (left ventricular wall thickness greater than or equal to 30 mm, nonsustained ventricular tachycardia on Holter ECG) for sudden death in HCM, and was considered to have a class IIa indication for ICD implantation. We placed ICD (Atlas™ + DR, St Jude Medical), which subsequently terminated several ventricular tachycardia episodes with anti-tachycardia pacing.

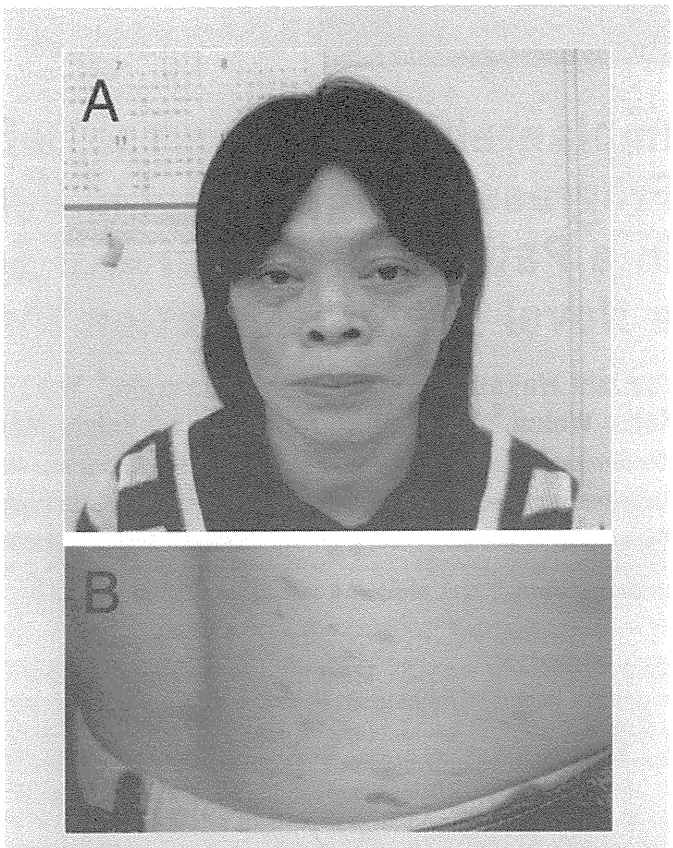

FIG. 1. Photographs of the patient at age 37 years. A: Craniofacial features. Hypertelorism, prominent eyes, a flat nose with anteverted nostrils, low-set posteriorly rotated ears, a long philtrum, thick lips, and multiple lentigines are noted, but typical adult Noonan syndrome-like features such as an inverted triangular shape with a pointed chin, a pinched nasal root, and prominent nasolabial folds are lacking. B: Café-au-lait spots on the back.

MUTATION ANALYSIS

Genomic DNA was isolated from the peripheral blood leukocytes of the patient. Each exon with flanking intronic sequences in *PTPN11* was amplified by polymerase chain reaction (PCR) with primers based on GenBank sequences. The primer sequences are available on request. PCR amplification was performed under standard condition using Taq DNA polymerase. After amplification, the PCR products were gel-purified and sequenced on an ABI PRISM 310 automated DNA sequencer (Applied Biosystems, California). A heterozygous missense mutation (c. 1,530 G > C; p. Gln510His) was identified in exon 13 (data not shown).

TABLE I. Patients With Mutations at Codon 510 of *PTPN11*

Family	1			2			3	4	5	6	7	8
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Mutation		Gln510Pro			Gln510Pro		Gln510Glu	Gln510Glu	Gln510Glu	Gln510Glu	Gln510Glu	Gln510His
Sex	F	M	F	F	F	M	M	F	M	M	F	F
Age at publication (y, years; m, months)	?	12y	25y	?	?	4y	1y 3m	2y	2.3y	2m	37y	38y
Lentigines	+	+	+	+	+	−[a]	−	+	−	−	+	+
Café-au-lait spots	−	+	+					+	+	−		+
Congenital heart defects	−	+ (non-PS)	+ (PS, MVP)	−		+ (PS, ASD)	−	+ (MVA)	+ (PS)	+ (MR, VSD)	−	+ (PS, ASD)
Cardiomyopathy	−	−	−				HCM	HCM	HCM	HCM	HCM	HCM
ECG conduction abnormalities	?	+	−	+	−	+					+	+
Hypertelorism	−	−	−	+?	+	+	+	+	−	+	+	+
Prominent eyes									+	+		+
Ptosis	−	−	−					+	+			−
Low-set ears						+	+	+		+		+
Dysmorphic ears				+?	+	+	+	+	+	+		+
Hearing impairment	−	+	+	−	+	−	+	−	−	−	+	−
Genital abnormalities	−	C	−	−	−	−				C		
Scoliosis	−	−	−	−	+	−						
Coagulation abnormalies	−	+	+									
Growth retardation	−	−	−	+	−	+	−	+	+	−	+	+
Mental retardation	−	−	−			MDD	MDD	MDD	MDD			+
References	Keren et al. [2004]			Kalidas et al. [2005]			Takahashi et al. [2005]	Digilio et al. [2006]		Faienza et al. [2009]	Lehmann et al. [2009]	Present patient

Patient 1 was the mother of Patient 2 and Patient 3. Patient 4 and Patient 5 were the maternal grandmother and mother of Patient 6, respectively.
F, female; M, male; +, present; −, absent.
PS, pulmonary stenosis; MVP, mitral valve prolapse; ASD, atrial septal defect; MVA, mitral valve anomaly; MR, mitral valve regurgitation; VSD, ventricular septal defect; HCM, hypertrophic cardiomyopathy; C, cryptorchidism; MDD, motor developmental delay.
[a]absent at age 1 year.

DISCUSSION

This patient fulfills the clinical diagnostic criteria of LS proposed by Voron et al. [1976] with a novel heterozygous mutation Gln510His in *PTPN11*, the major causative gene for LS. *PTPN11* mutations in patients with LS are clustered in exons coding the protein tyrosine phosphatase domain, with two recurrent mutations in exons 7 (Tyr279Cys) and 12 (Thr468Met) in about 65% of *PTPN11*-positive cases, and other rare mutations [Digilio et al., 2002; Sarkozy et al., 2008]. Heterozygous missense mutations at codon 510 in exon 13 have been reported in 12 patients from eight families including this patient (Table I) [Keren et al., 2004; Kalidas et al., 2005; Takahashi et al., 2005; Digilio et al., 2006; Faienza et al., 2009; Lehmann et al., 2009]. A Gln510Glu mutation was found in five sporadic patients, who all manifested HCM with or without congenital heart defects. HCM was detected prenatally in two patients [Digilio et al., 2006], on the first day of life in one [Faienza et al., 2009], at age 1 month in one [Takahashi et al., 2005], and at age 23 years in one [Lehmann et al., 2009]. Pharmacotherapy including diuretics and propranolol was effective in two patients with progressive HCM with left ventricular outflow tract obstruction and congestive heart failure [Takahashi et al., 2005; Digilio et al., 2006; Limongelli et al., 2008]. Septal myectomy was required in one [Digilio et al., 2006; Limongelli et al., 2008] and sudden death occurred in one [Faienza et al., 2009]. On the other hand, a Gln510Pro mutation was found in six patients from two families, none of whom was described to manifest HCM, though three had congenital heart defects and two were elders at publication [Keren et al., 2004; Kalidas et al., 2005]. Limongelli et al. [2008] reviewed 24 LS patients with ($n = 16$) and without ($n = 8$) *PTPN11* mutations. They proposed mutations in exon 13 and codon 510 as molecular predictors of adverse cardiac events (life-threatening arrhythmic events, cardiac arrest, and heart failure), as well as LVH at ECG, New York Heart Association class >2, maximal wall thickness z-score $> +10$, LVOT gradient >50 mmHg, and NSVT as clinical predictors of these events. However, six patients from two families with a Gln510Pro mutation did not show HCM (Table I) [Keren et al., 2004; Kalidas et al., 2005]. Thus, presence of specific missense mutations at codon 510 (Gln510Glu and Gln510His, not Gln510Pro) would be a molecular risk factor of adverse cardiac events. The boys described by Takahashi et al. [2005] and Faienza et al. [2009] were diagnosed with Noonan syndrome because of no pigmented spots at the time of publication. They might develop lentigines and be diagnosed with LS, like the family described by Kalidas et al. [2005] (the 4-year-old boy showed no lentigines, while his mother and grandmother with the same mutation showed multiple lentigines).

Management of each "RASopathy" might depend on the cardiac phenotype. Whereas pulmonary valve stenosis with dysplastic leaflets and atrial/ventricular septal defects are the most prevalent cardiac defects in patients with Noonan syndrome caused by gain-of-function mutations in *PTPN11*, HCM is the most frequent cardiac complication and represents the only life-threatening problem in patients with LS caused by dominant-negative mutations in *PTPN11* [Sarkozy et al., 2008; Marin et al., 2011]. Indeed, the present patient could return to work under an appropriate cardiac management including intensive pharmacotherapy for controlling heart failure and ICD for preventing fatal arrhythmias. HCM in LS patients, which in general is asymptomatic and involves the left ventricle, is complicated by left ventricular outflow tract obstruction in up to 40% of the cases and frequently manifests during the second infancy before multiple lentigines occur [Sarkozy et al., 2008]. Therefore, those with LS, as well as those clinically diagnosed with Noonan syndrome and having HCM, are recommended to have molecular testing of *PTPN11* for genotype-based risk stratification of fatal cardiac events. LS patients with symptomatic HCM should receive intensive pharmacotherapy including beta blockers, calcium channel blockers, digoxin, diuretics, antiarrhythmic drugs, and angiotensin-converting enzyme inhibitors, depending on their symptoms and cardiac features; and for drug-refractory patients with obstructive HCM, surgical relief of left ventricular outflow obstruction is considered [Maron et al., 2003; Biagas and Hsu, 2006]. LS patients with symptomatic or asymptomatic HCM are recommended to have regular cardiac ultrasonography to measure left ventricular wall thickness and Holter ECG to detect nonsustained ventricular tachycardia for an indication of ICD implantation. Furthermore, etiology-based therapy might be realized, as recently published study by Marin et al. [2011], proposing effectiveness of TOR inhibitors such as rapamycin for the treatment of HCM in LS patients based on an evidence that dominant-negative *PTPN11* mutations in LS would enhance mTOR activity as critical for causing LS-associated HCM in a mouse model.

In conclusion, we have reported successful intervention through ICD implantation on a woman with LS and progressive HCM accompanied by congestive heart failure and nonsustained ventricular tachycardia, who was found to have a novel Gln510His mutation in *PTPN11*. Review of patients with mutations at codon 510 in *PTPN11* suggested that specific mutations (Gln510Glu, Gln510His, not Gln510Pro) would be a predictor of fatal cardiac events in LS. Molecular risk stratification and careful evaluations for an indication of ICD implantation are likely to be beneficial in managing patients with LS and HCM. Continued molecular characterization with cardiac phenotypes of these patients is crucial in further delineation of the risks as well as future etiology-based therapy.

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Case report

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

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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

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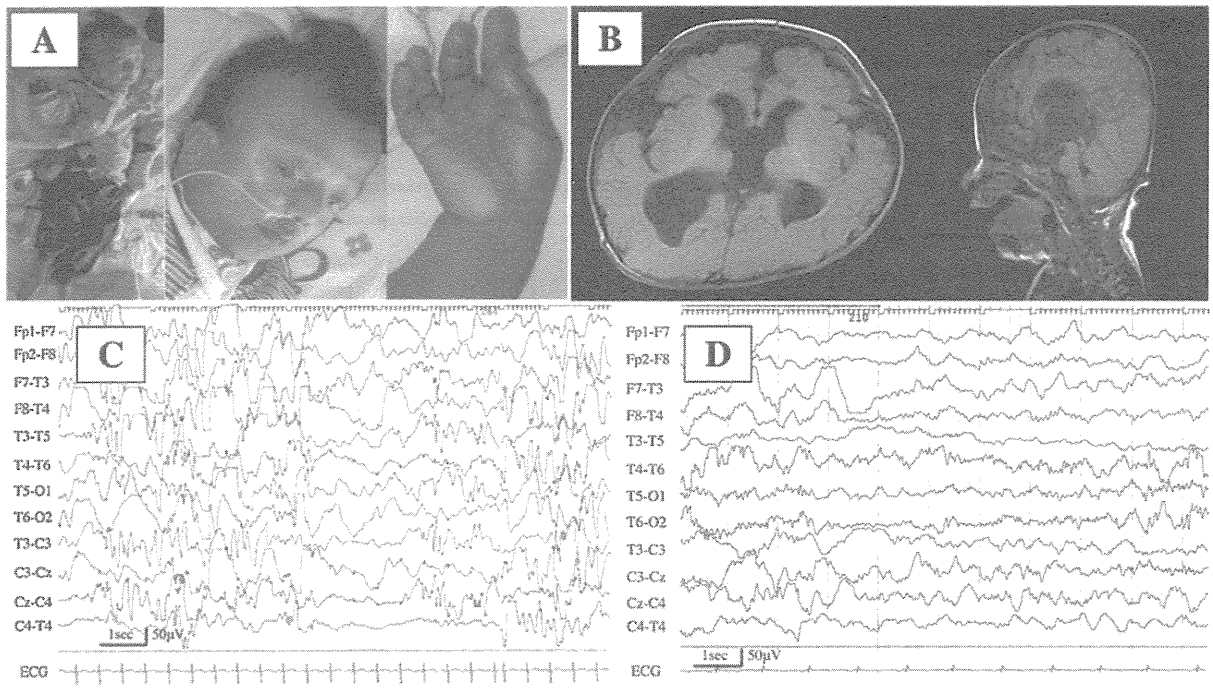

Fig. 1. Case 1. "Coarse face," curly hair, prominent forehead, dysmorphic ears, abnormal loose and pigmented skin, webbed neck, chest deformity, and micromelic dwarfism at six years (A), agenesis of the corpus callosum, ventricular dilatation, diffuse cortical atrophy and severely delayed myelination 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,

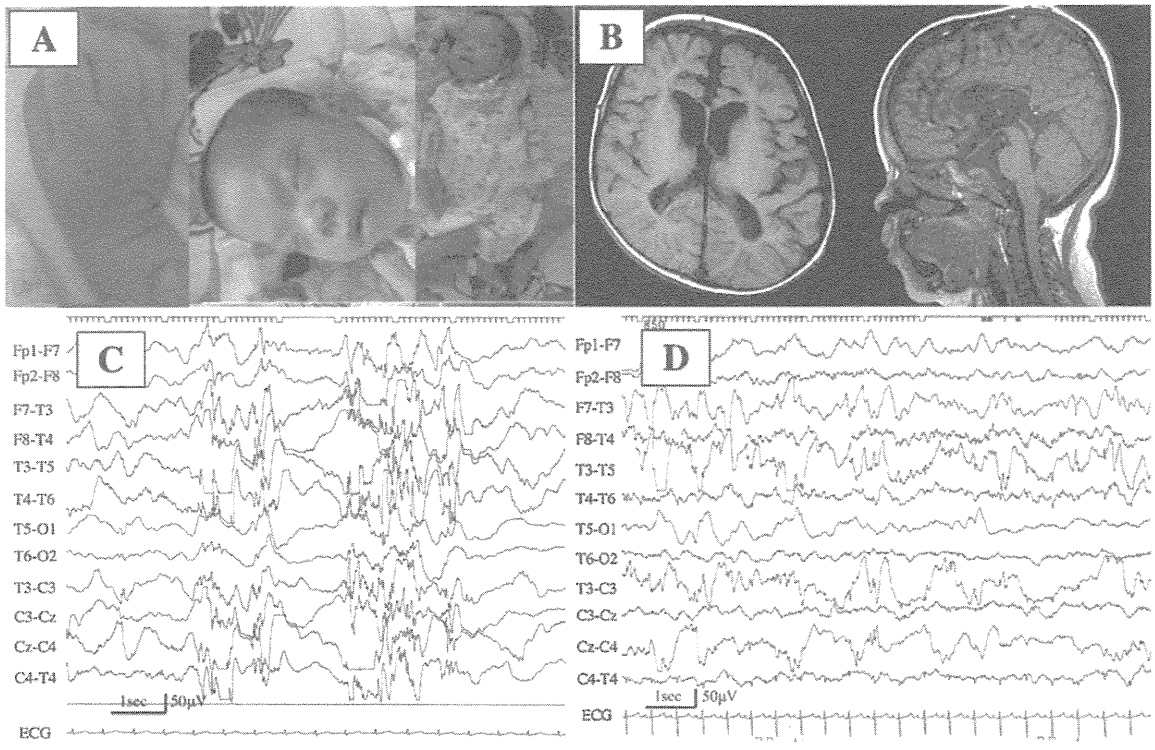

Fig. 2. Case 2. Odd looking "coarse" face (prominent forehead, short nose, and low-set dysmorphic ears, curly sparse hair), abnormal loose, pigmented skin with multiple lentigo, narrow chest, and hypotonic micromelic dwarfism at four years (A), diffuse brain atrophy with delayed myelination and hypoplastic 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.

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