Molecular characterization of folate receptor 1 mutations delineates cerebral folate transport deficiency

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Cerebral folate transport deficiency is an inherited brain-specific folate transport defect that is caused by mutations in the folate receptor 1 gene coding for folate receptor alpha (FRα). This genetic defect gives rise to a progressive neurological disorder with late infantile onset. We screened 72 children with low 5-methyltetrahydrofolate concentrations in the cerebrospinal fluid and neurological symptoms that developed after infancy. We identified nucleotide alterations in the folate receptor 1 gene in 10 individuals who shared developmental regression, ataxia, profound cerebral hypomyelination and cerebellar atrophy. We found four novel pathogenic alleles, one splice mutation and three missense mutations. Heterologous expression of the missense mutations, including previously described mutants, revealed minor decrease in protein expression but loss of cell surface localization, mistargeting to intracellular compartments and thus absence of cellular binding of folic acid. These results explain the functional loss of folate receptor alpha for all detected folate receptor 1 mutations. Three individuals presenting a milder clinical phenotype revealed very similar biochemical and brain imaging data but partially shared pathogenic alleles with more severely affected patients. Thus, our studies suggest that different clinical severities do not necessarily correlate with residual function of folate receptor alpha mutants and indicate that additional factors contribute to the clinical phenotype in cerebral folate transport deficiency.

Keywords: cerebral folate transport deficiency; cerebral folate deficiency; folate receptor; neurodegeneration; folate
Abbreviations: FOLR1 = folate receptor 1; FRα = folate receptor alpha; 5-MTHF = 5-methyltetrahydrofolate

Introduction

Folates are essential for a variety of biological processes involving the transfer of methyl groups to amino acids, nucleotides, neurotransmitters and phospholipids. Commonly, the methylation is mediated by S-adenosyl methionine as methyl group donor that is in turn regenerated by 5-methyltetrahydrofolate (5-MTHF), the predominant physiological form of folate (Antony, 1992; Kamen and Smith, 2004; Ramaekers and Blau, 2004). 5-MTHF is actively transported across the blood–CSF barrier by an unclarified
mechanism. However, mutations in the folate receptor 1 (FOLR1) gene are associated with severely reduced concentrations of 5-MTHF in the CSF indicating that its gene product, folate receptor alpha (FRα), plays a crucial role in this transport process (Steinfeld et al., 2009).

This FRα defect causes cerebral folate transport deficiency (MIM 136430), a progressive neurological disorder with late infantile onset that is characterized by psychomotor regression, epilepsy and disturbed brain myelination as well as a depletion of white matter choline and often inositol (Cario et al., 2009; Steinfeld, et al., 2009; Perez-Duenas, et al., 2010; Dill et al., 2011). Folinic acid therapy restores glial choline and inositol content, leads to normalization of 5-MTHF concentration in the CSF and ameliorates the symptoms in most patients.

FRα is a glycosylphosphatidylinositol-anchored receptor with high affinity for serum folate in the nanomolar range ($K_d = 0.4 \text{ nmol/l}$ for folic acid and $3 \text{ nmol/l}$ for 5-MTHF; Doucette and Stevens, 2004) and is expressed at the surface of epithelial cells (Weitman et al., 1992a, b; Steinfeld et al., 2009). In human brain, FRα is preferentially expressed in the choroid plexus, indicating that the major supply route for brain 5-MTHF occurs via the blood–CSF barrier (Ramaekers and Blau, 2004; Steinfeld et al., 2009; Hyland et al., 2010).

At least six distinct inherited disorders of folate metabolism and transport are presently known, most of which lead to generalized folate deficiency (Goyette et al., 1994; Leclerc et al., 1996, 1998; Rosenblatt and Fenton, 2001; Hilton et al., 2003; Qu et al., 2006; Zhao et al., 2009; Banka et al., 2011; Cario et al., 2011). In addition, cerebral folate deficiency might be caused by autoantibodies against folate receptors (Ramaekers et al., 2005).

Our present work confirms and extends the clinical and pathogenetic spectrum of cerebral folate transport deficiency and further explains its molecular mechanism.

**Materials and methods**

**Patients**

Data and samples from patients with the clinical features of cerebral folate transport deficiency were transferred from various genetic and neuropaediatric clinics in Germany, Finland, Italy, Switzerland, The Netherlands, UK and the USA over a period of 2 years. Among the investigated families were several of Turkish, Arabian, Azerbaijani and Kurdish origin with a high percentage of consanguineous marriages. Metabolic work-up of patients excluded abnormalities in the full blood count, plasma amino acid concentrations including plasma homocystein concentration, urinary organic acids and plasma folate and vitamin $B_12$ concentrations. Patients were recruited when the 5-MTHF concentration in the CSF was $<40 \text{ nmol/l}$. CSF 5-MTHF concentrations $\leq 5 \text{ nmol/l}$ were classified as extremely low and were found in all patients carrying FOLR1 mutations (Table 1). Informed consent was obtained from the parents, and the study was approved by the ethical review board of the medical faculty of the University of Göttingen. Clinical and biochemical data from Patient 11 were previously published (Perez-Duenas et al., 2010). Minor clinical data from Patients 8, 9 and 10 were mentioned in the initial description of cerebral folate transport deficiency (Steinfeld et al., 2009). Patient 6 is compound heterozygous for the missense mutations R722H and V1044A in the POLG1 gene. Though the pathogenicity of these mutations is not proven, we cannot exclude that they contribute to the severe phenotype of this patient (Ishoanni et al., 2011).

**Mutation screening**

Genomic DNA was extracted from peripheral blood leukocytes using the NucleoSpin® Blood L kit (Macherey-Nagel). The FOLR1 gene was analysed by direct sequencing of PCR products using the BigDye™ Terminator Ready Reaction chemistry on the ABI Prism® 3100-Avant Genetic Analyser (Applied Biosystems). Primer sequences as well as PCR and sequencing conditions are available upon request. Nucleotide positions are numbered with $+1$ as the A of the ATG translation codon based on the reference sequence of the complementary DNA (Genbank NM_016725.2). The mutation nomenclature follows guidelines recommended on the Mutation Nomenclature Homepage at the HGVS website (http://www.hgvs.org/mutnomen/). Novel point mutations detected by sequencing were confirmed by following the Mendelian transmission in parents and other members of the family. All previously unreported FOLR1 mutations were excluded to be present in 200 alleles of 100 unaffected controls by direct sequencing.

**Expression of wild-type and mutants of folate receptor alpha**

Cloning of FRα complementary DNA has been described previously (Steinfeld et al., 2009). Mutations were introduced into human FRα cloned into the expression vector pcDNA3.1(−) (Invitrogen) by PCR-mediated *in vitro* mutagenesis according to the manufacturer’s instructions for QuikChange® Site-Directed Mutagenesis kit (Stratagene). Chinese hamster ovary (CHO-K1) cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO₂. The human polarized hepatic cell line HepG2 was grown in RPMI 1640 supplemented with 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO₂. Transfection of the expression plasmids was done using Effectene® (QiAGEN) according to the manufacturer’s protocol. Stable clones were selected in the presence of G418 (800 μg/ml).

**Folic acid binding**

Stable transfected CHO-K1 or HepG2 cells were seeded in 6-well plates 48 h before the experiment. For transient transfections, CHO-K1 cells were seeded 3 days before the experiment and transfected on the second day. Cells were then washed with ice-cold PBS, followed by a washing step with ice-cold acid buffer [10 mmol/l NaAC, 150 mmol/l NaCl (pH 3.5)] to release surface bound folate. After a washing step with ice-cold Hank’s balanced salt [20 mmol/l HEPES, 140 mmol/l NaCl, 5 mmol/l KCl, 2 mmol/l MgCl₂, 5 mmol/l glucose (pH 7.4)], cells were exposed for 15 min to 5 mmol/l [$^3$H]folic acid (Moravek Biochemicals) in the absence or presence of 500 nmol/l non-labelled folic acid. After three ice-cold Hank’s balanced salt washes, cell surface bound [$^3$H]folic acid was released with acid buffer (1 ml) and measured on a liquid scintillation spectrometer. Specific folic acid binding was calculated from the difference between [$^3$H]folic acid bound in the presence and absence of 500 nmol/l non-labelled folic acid. The adherent cells were subsequently lysed and protein was determined by using the BCA protein kit (Pierce).
Table 1: Clinical, biochemical and neuroradiological data of patients with genetically confirmed cerebral folate transport deficiency

<table>
<thead>
<tr>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
<th>Patient 7</th>
<th>Patient 8</th>
<th>Patient 9</th>
<th>Patient 10</th>
<th>Patient 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex/ethnic background</td>
<td>Female/Finnish</td>
<td>Male/Azeri</td>
<td>Female/Finnish</td>
<td>Female/Finnish</td>
<td>Male/Finnish</td>
<td>Female/Finnish</td>
<td>Male/Turkish</td>
<td>Female/Italian</td>
<td>Male/Italian</td>
<td>Male/German</td>
</tr>
<tr>
<td>CSF 5-MTHF concentration</td>
<td>&lt;5 nmol/l</td>
<td>3 nmol/l</td>
<td>5 nmol/l</td>
<td>&lt; 5 nmol/l</td>
<td>5 nmol/l</td>
<td>&lt; 5 nmol/l</td>
<td>&lt; 5 nmol/l</td>
<td>1.4 nmol/l</td>
<td>2 nmol/l</td>
<td>2 nmol/l</td>
</tr>
<tr>
<td>Age of onset and initial symptoms</td>
<td>1 year, motor developmental delay</td>
<td>22 months seizure, developmental delay</td>
<td>Congenital microcephaly, 3 months, hypotonia, ataxia</td>
<td>2 years, ataxia</td>
<td>15 years, motor developmental delay</td>
<td>2 years, global developmental delay</td>
<td>2 years, speech delay</td>
<td>2.5 years, motor deficit, ataxia</td>
<td>2.5 years, tremor, slight ataxia</td>
<td>2nd year, progressive ataxia, tremor in upper limbs</td>
</tr>
<tr>
<td>Head circumference (age)</td>
<td>0 SD at birth, 1 cm below 3rd percentile (6 years)</td>
<td>Below 3rd percentile (3 months), 47 cm at 12 years</td>
<td>Severe</td>
<td>Moderate</td>
<td>Severe</td>
<td>Moderate</td>
<td>Severe</td>
<td>Moderate</td>
<td>Severe</td>
<td>None</td>
</tr>
<tr>
<td>Developmental regression</td>
<td>Severe</td>
<td>Severe</td>
<td>Severe</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Severe</td>
<td>Moderate</td>
<td>Severe</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
<tr>
<td>Motor deficits</td>
<td>Hypotonia, ataxia, ataxia, ataxia</td>
<td>Hypotonia, ataxia, ataxia, ataxia</td>
<td>Hypotonia, ataxia, ataxia, ataxia</td>
<td>Hypotonia, ataxia, ataxia, ataxia</td>
<td>Hypotonia, ataxia, ataxia, ataxia</td>
<td>Hypotonia, ataxia, ataxia, ataxia</td>
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<td>Hypotonia, ataxia, ataxia, ataxia</td>
<td>Hypotonia, ataxia, ataxia, ataxia</td>
</tr>
<tr>
<td>Lower limb pyramidal tract signs</td>
<td>Hyperreflexia, Babinski sign, distal spasticity (4 years)</td>
<td>None</td>
<td>Hyperreflexia, Babinski sign, distal spasticity (4 years)</td>
<td>None</td>
<td>Hyperreflexia, Babinski sign, distal spasticity (4 years)</td>
<td>None</td>
<td>Hyperreflexia, Babinski sign, distal spasticity (4 years)</td>
<td>None</td>
<td>Hyperreflexia, Babinski sign, distal spasticity (4 years)</td>
<td>None</td>
</tr>
<tr>
<td>Autistic behaviour</td>
<td>Autistic signs</td>
<td>None</td>
<td>Autistic signs</td>
<td>None</td>
<td>Autistic signs</td>
<td>None</td>
<td>Autistic signs</td>
<td>None</td>
<td>Autistic signs</td>
<td>None</td>
</tr>
<tr>
<td>Cranial MRI abnormalities</td>
<td>Delayed myelination, T2-hyperintensity of the periventricular and subcortical white matter lesions</td>
<td>Delayed myelination, T2-hyperintensity of the periventricular and subcortical white matter lesions</td>
<td>Delayed myelination, T2-hyperintensity of the periventricular and subcortical white matter lesions</td>
<td>Irregular myelination, cerebellar and cerebral atrophy</td>
<td>Hypomyelination cerebellar and cerebral atrophy, focally T2-hyperintense white matter lesions</td>
<td>Hypomyelination cerebellar and cerebral atrophy, focal T2-hyperintense white matter lesions</td>
<td>Hypomyelination cerebellar and cerebral atrophy, focal T2-hyperintense white matter lesions</td>
<td>Hypomyelination cerebellar and cerebral atrophy, focal T2-hyperintense white matter lesions</td>
<td>Hypomyelination cerebellar and cerebral atrophy, focal T2-hyperintense white matter lesions</td>
<td>Hypomyelination cerebellar and cerebral atrophy, focal T2-hyperintense white matter lesions</td>
</tr>
<tr>
<td>MRS abnormalities</td>
<td>Low choline, normal myoinositol</td>
<td>Normal</td>
<td>Low choline, normal myoinositol</td>
<td>Normal</td>
<td>Normal</td>
<td>Very low choline, low inositol</td>
<td>Very low choline, low inositol</td>
<td>Very low choline, low inositol</td>
<td>Very low choline, low inositol</td>
<td>Very low choline</td>
</tr>
</tbody>
</table>

(continued)
**Immunoblot analysis**

CHO-K1 or HepG2 cells stably expressing wild-type or mutant FRα were grown on coverslips to 80% confluence. The cells were washed twice with PBS and were fixed with 4% (w/v) paraformaldehyde for 20 min. Free aldehyde groups were quenched in 50 mM NH₄Cl for 10 min at room temperature. Cells were washed again three times with PBS and then permeabilized with 5 μl lysis buffer per 100 mm dish for 30 min on ice. Lysates were then centrifuged at 18 000g for 10 min at 4°C and 80 μg of protein were denatured in Laemmli sample buffer with 5% (v/v) β-mercaptoethanol. Immunoblot analysis was followed up as described above.

**Immunofluorescence microscopy**

CHO-K1 cells stably expressing wild-type or mutant FRα were grown on coverslips and incubated with paraformaldehyde for 20 min. Free aldehyde groups were quenched in 50 mM NH₄Cl for 10 min at room temperature. Subsequently, the permeabilized cells were incubated with primary antibodies, mouse monoclonal anti-human FRα antibody (Mov18; 1:100 dilution) and rabbit polyclonal anti-PDI (1:250 dilution, Sigma). Cells were incubated with the appropriate secondary antibodies, mouse monoclonal anti-human FRα and Alexa Fluor 488 or 546 for 1 h in the dark. After additional three washing steps with PBS, coverslips were mounted on object glasses with ProLong Gold (Invitrogen). Images were acquired using a spinning disk confocal imaging system (UltraView, PerkinElmer). The detection system comprised an Olympus IX81 inverted microscope using a ×100 1.4 NA lens. At least 50 cells per coverslip were examined under the microscope, and representative cells were photographed.

**Reverse transcription-polymerase chain reaction analysis**

Reverse transcription PCR was used to analyse deduced splicing-defects on messenger RNA level in cultured fibroblasts from a patient and controls, respectively. RNA was isolated from fibroblasts with the RNAasy Kit as described in a separate report. Complementary DNA synthesis, the Superscript III First-Strand Synthesis System (Invitrogen) was used. Specific primer pairs for FOLR1 complementary DNA were FR1cDNA_49F (5'-gctgtagtgagggagagt-3') and FR1cDNA_363F (5'-atccacgtggtcagcag-3'), FR1cDNA_363F (5'-ggcagcagcaggagcc-3') and FR1cDNA_475F (5'-ggctgaggatggtcagcag-3') and FR1cDNA_711R (5'-ggctgtagtgagggagagt-3'). PCR amplification was carried out using 0.5 μl of complementary DNA with 37 cycles and 60°C annealing temperature. Reverse transcription PCR products were analysed by agarose gel electrophoresis.
Results

Phenotypic characteristics of patients suffering from cerebral folate transport deficiency

From a total of 72 neuropaediatric patients with low 5-MTHF concentration in the CSF we identified 10 patients with mutations in the FOLR1 gene. However, within the group of 14 patients with extremely low 5-MTHF, i.e., \( \leq 5 \) nmol/l, 71% were positive for FOLR1 mutations (Table 1) and only four patients carried no pathogenic alteration in the FOLR1 gene. In these four patients, we excluded pathogenic mutations in the coding regions of the following other folate transporter genes: folate receptor 2, reduced folate carrier and proton-coupled folate transporter.

All patients suffering from cerebral folate transport deficiency presented initial symptoms such as developmental delay, ataxia and cerebral seizures within the first 3 years of life and most of them developed motor deficits as well as behavioural abnormalities. However, the frequency and severity of cerebral seizures as well as the degree of developmental regression varies among patients. In particular, Patients 4, 5 and 7 were only moderately developmentally retarded and less affected by epileptic seizures. Other characteristic features shared by all patients include a slow background rhythm and multifocal epileptiform activity in the EEG. The cranial MRIs most frequently show delayed myelination or hypomyelination of the cerebral white matter and a slight cerebral but more pronounced cerebellar atrophy (Fig. 1). The two oldest patients, Patients 3 and 4, developed a severe polyneuropathy at 15 and 13 years of age, respectively. This represents a late finding, but may be an important sign in other patients as well.

Molecular genetic analysis of the FOLR1 mutations

Direct sequencing of genomic patient DNA resulted in the identification of four novel pathogenic alleles in the human FOLR1 gene in seven individuals. Three missense mutations p.C65W, p.C169Y, p.N222S were positioned in exon 5 and 7, respectively, and one splice mutation g.3576T>G affected the splice donor site of intron 6 (Fig. 2). To confirm the splice defect caused by the mutation g.3576T>G, we analysed the complementary DNA of the patient. Amplification of a complementary DNA fragment comprising exon 6–7 with the primer pair 475F and 711R resulted in larger fragments in the patient when compared with two controls. The increase in length of the major band by 150 bp corresponds to the size of intron 6 and hence is consistent with the defective splicing of intron 6. The amplification of other complementary DNA fragments gave equivalent banding for the patient and controls excluding the contamination with significant amounts of genomic DNA.

Detection of folate receptor alpha mutants in patient fibroblasts and expression of folate receptor alpha mutants in CHO-K1 and HepG2 cells

We first analysed the expression of FRα in patient fibroblast by immunoblot experiments (Fig. 3A). In fibroblast lysates from two controls, FRα protein migrates with a size of 38 kDa and corresponds to the fully maturated and processed form of FRα. In contrast, no expression was found for Patients 9 and 10 carrying the compound heterozygous mutations p.Q118X and p.C175X confirming our previous results that the two premature termination codons result in a complete lack of FRα expression (Steinfeld et al., 2009). For Patient 5 with the two heterozygous mutations p.C169Y and p.N222S, a major but slightly faster migrating species of FRα could be detected beside the 38 kDa form of lower intensity. The slightly smaller form of the protein might be due to a disturbed post-translational modification or maturation of FRα. For Patient 1 carrying the homozygous mutation p.C169Y, we could only detect a weak faster migrating band and no expression of the 38 kDa fully maturated FRα form. No expression of FRα could be detected in fibroblasts from Patient 7 with the splice site mutation g.3576T>G indicating that this mutation might lead to degradation of the mutant protein. Reduced expression of the 38 kDa form and appearance of the faster migrating species was also found for the previously described Patient 8 with the duplication p.K44_P49dup (Steinfeld et al., 2009).

We further analysed the functional consequences of the three novel missense mutations p.C65W, p.C169Y and p.N222S as well as of the two known mutants p.C105R (Perez-Duenas et al., 2010) and p.K44_P49dup in a heterologous cell expression system. To determine whether these mutations affect protein stability, immunoblot analysis of stable transfected CHO-K1 cells was performed (Fig. 3B). The wild-type and the mutant proteins p.C105R, p.C169Y, p.N222S and p.K44_P49dup were detected by the monoclonal anti-human FRα antibody NCL-L-FRalpha (Clone BN3.2, Novocastra) with similar intensities, indicating that the expression level of these mutant proteins is only slightly affected. In contrast, transfection of mutant p.C65W in CHO-K1 cells showed only an expression level of 8% compared with the wild-type protein. Densitometric analysis of the bands in immunoblots of three independent experiments supports this finding (Supplementary Fig. 1). Interestingly, none of the mutant proteins were detected by the monoclonal antibody against human FRα Mov18/ZEL (Alexis Biochemicals) indicating that this antibody reacts with an epitope that is critically dependent on the natural FRα conformation (Fig. 3B). Comparable results were obtained in immunoblot experiments with human polarized hepatic cells HepG2 stable expressing wild-type or mutants of FRα (Supplementary Fig. 2A).

Functional characterization of wild-type and mutants of folate receptor alpha by folic acid binding assay

Since protein expression of FRα mutants was not significantly altered, we further characterized their effect on folic acid binding
to CHO-K1 cells stably transfected with wild-type or mutants of FRα (Fig. 3C). Transfection of the FRα wild-type resulted in a 13-fold increase in folic acid binding compared with the vector-transfected cells. Folic acid binding of mutants p.C65W, p.C105R and p.C169Y was indistinguishable from vector-transfected cells, but transfection of p.N222S resulted in a slightly increased folic acid binding by 2.5-fold. Similar results were obtained in folic acid binding experiments with HepG2 cells stable expressing FRα wild-type or mutants p.C169Y and p.N222S (Supplementary Fig. 2B).

Figure 1 Brain imaging of patients with genetically confirmed cerebral folate transport deficiency. Shown are sagittal T1-weighted magnetic resonance scans (T1sag) as well as axial T2-weighted magnetic resonance images (T2ax) for all 10 patients that are described and numbered in Table 1. Note that in case of Patient 2 a sagittal T1-weighted MRI with contrast medium (T1sag + CM) is depicted. Patient number and age is indicated in the lower middle area of the pictures.

Mistargeting of folate receptor alpha mutants expressed in CHO-K1 cells

FRα mutants exhibit severely reduced folic acid binding while their protein expression is little altered indicating that missorting of FRα mutants might cause loss of cell surface binding. To prove this hypothesis, we investigated the cellular targeting of FRα wild-type and mutants in CHO-K1 cells by confocal immunofluorescence microscopy. Whereas, FRα wild-type localized predominantly to
the plasma membrane, the mutants p.C65W, p.C105R, p.C169Y and p.N222S were mistargeted to intracellular compartments and partially colocalized with the endoplasmic reticulum marker protein disulphide isomerase (Fig. 4).

Discussion

The present study has elucidated common clinical, biochemical and molecular features for patients with cerebral folate transport deficiency (Supplementary Table 1). All patients share an extremely low 5-MTHF concentration in the CSF, presented first symptoms by the age of 3 years, revealed developmental regression and ataxia, showed delayed myelination of the CNS and cerebellar atrophy and disclosed a slow background rhythm with multifocal epileptiform activity in the EEG. Most patients suffer from frequent myoclonic seizures, but may also present astatic and tonic, brief involuntary, symmetric contractions of the neck and trunk reminiscent of infantile spasm that usually last <1 min. Focal T2-hyperintense lesions of the subcortical or periventricular
Figure 3 Detection of FRα mutants in patient fibroblasts, heterologous expression and functional characterization of wild-type (WT) and mutant FRα in CHO-K1 cells. 

(A) Immunoblot analysis of endogenous FRα in patient fibroblasts. Protein (80 μg) from fibroblast cell lysates was separated by SDS–PAGE on a 12% acrylamide gel. FRα was detected using the mouse monoclonal anti-human FRα antibody NCL-L-FRalpha. β-Actin served as a loading control. FRα missense mutants (p.C169Y, p.N222S and p.K44_P49dup) revealed species of higher mobility and showed a reduced level of expression of the mature 38 kDa FRα wild-type. FRα stop and splice mutants (p.Q118X, p.C175X and g.3576T>G) gave no detectable expression. 

(B) Immunoblot analysis from CHO-K1 cells stable transfected with wild-type or mutant FRα. Samples were prepared as described above and 60 μg of protein were separated by SDS–PAGE on a 12% acrylamide gel. Immunodetection was carried out using the FRα antibodies NCL-L-FRalpha and Mov18/ZEL. All depicted FRα missense mutants could be detected by the antibody NCL-L-FRalpha, however the intensity of the mutant p.C65W was only 8% of the FRα wild-type band. In contrast, none of the FRα missense mutants could be detected by the antibody Mov18/ZEL. 

(C) Folic acid surface binding by CHO-K1 cells stably transfected with wild-type or mutant FRα. Cells were exposed to 5 nmol/l [3H]folic acid for 15 min at pH 7.4. Values are means ± S.D. from three independent experiments, each carried out in duplicate. When compared with vector-transfected CHO-K1, only mutant N222S revealed a slight (2.5-fold) increase in folic acid binding. All other mutants (p.C65W, p.C105R, p.C169Y) failed to increase folic acid binding.

Figure 4 Subcellular localization of wild-type (WT) and mutant FRα in CHO-K1 cells. CHO-K1 cells stable transfected with FRα wild-type and the mutants p.C105R, p.C169Y, p.N222S and p.C65W were immunolabelled with mouse monoclonal antibody against human FRα Mov18/ZEL (red) and a rabbit polyclonal antibody against the endoplasmic reticulum (ER) protein disulphide isomerase (green) followed by confocal immunofluorescence microscopy. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). FRα wild-type localized to the plasma membrane, whereas all three mutants were mistargeted to intracellular compartments that partially co-localized with the endoplasmic reticulum marker protein disulphide isomerase. Scale bars = 10 μm.
white matter are often found in cranial MRIs of older patients and most likely represent gliotic areas. Prognostic factors, associated with a milder phenotype include late-onset and infrequent epileptic seizures, are found in Patients 4 and 7 and to a lesser extent in Patient 5. The symptoms and their frequency described are instructive to discriminate FOLR1 mutation positive patients from other disease entities associated with cerebral folate deficiency (Mangold et al., 2011). Normal red blood count and normal plasma concentration of homocystein and folate exclude the presence of nutritional folate deficiency, hereditary folate malabsorption, methylenetetrahydrofolate reductase and dihydrofolate reductase deficiency. Other disorders associated with cerebral folate deficiency, such as Kearns–Sayre syndrome, Aicardi–Goutière’s syndrome, aromatic l-amino acid decarboxylase deficiency and serine biosynthesis defects commonly show CSF 5-MTHF >5 nmol/l and present various clinical signs that allow a convincing discrimination from cerebral folate transport deficiency. Based on the assumption that alternative or residual transport pathways into the brain can be exploited by increased plasma 5-MTHF concentrations, most patients have been treated with daily 2–5 mg folinic acid per kg body weight. Folinic acid (5-formyltetrahydrofolate) is preferred to folate (folic acid) since the latter must be converted by several enzymatic reactions to biological active 5-MTHF and it binds more strongly to FRα and thus inhibits binding of 5-MTHF to FRα. Most patients benefit from oral folinic acid treatment and respond within 2 months by reduced frequency of epileptic seizure as well as improved motor skills and show an increase of the 5-MTHF concentration in the CSF. The two oldest patients, Patients 3 and 4, were diagnosed at the age of 15 and 14 years, respectively and also responded to folinic acid therapy. Only Patient 6 did not respond to oral folinic acid treatment but this case could be aggravated by an additional mitochondrial affection (see ‘Patients’ section, above). Alternative routes of folinic acid supplementation have been tried in individual patients and preliminary results indicate that intravenous and/or intrathecal application may be superior to oral one. Patient 10, the sister of Patient 9, was treated with oral (5 mg/kg/day) and intravenous (100 mg once per week) folinic acid at the very beginning of the initial symptoms, and recovered completely and has not developed any neurological symptoms to date. Her cranial MRI only reveals a slight cerebellar atrophy but furthermore a completely normal cerebrum (Fig. 1). Thus, early treatment may prevent brain abnormalities and the development of severe neurological symptoms associated with this genetic defect.

We additionally investigated protein stability, folate binding and intracellular trafficking of mutant FRα proteins in heterologous expression systems. The stop mutations p.Q118X and p.C175X as well as the splice mutation g.3576T>G go along with a failure of FRα expression. In the latter, the base alteration affects the highly conserved splice donor site of intron 6 and thus results in reading through of intron 6 with an altered reading frame. After serine 164 this transcript can be translated to an additional 23 amino acids before a stop codon TGA occurs. Hence the splice mutation g.3576T>G leads to a truncated FRα mutant. Since all truncated FRα proteins lack serine 234 to which the glycosylphosphatidylinositol anchor is attached, these mutants fail membrane localization and result in complete loss of FRα function. In contrast, all missense mutants of FRα could be detected in patient fibroblasts and could be significantly expressed in CHO-K1 or HepG2 cells. The only exception being the mutant p.C65W, which showed an expression reduced to 8% of wild-type level (Fig. 3B). One can speculate that the elimination of cysteine 65 might cause the disruption of a structurally important disulfide bond and hence result in an instable conformation of FRα. The increased mobility of p.C169Y in patient fibroblasts (Fig. 3A) also suggests a structural alteration of this mutant that might be explained by a loss of disulfide bonding or disturbed post-translational processing. The highest protein expression level was found in fibroblasts with the genotype p.C169Y/p.N222S. In addition, our molecular experiments revealed a slight folate acid binding in the case of mutant p.N222S only. One is tempted to explain the less severe phenotype of Patient 5, which is compound heterozygous for p.C169Y/p.N222S, by these experimental findings. However, two other patients, Patients 4 and 7, presenting an even milder phenotype, carried a homozygous FRα mutant p.C169Y with no detectable residual folate acid binding (Fig. 3C) and the splice mutation g.3576T>G with no detectable expression of FRα in patient fibroblasts (Fig. 3A), respectively. In addition, Patients 1, 3 and 6 share the same homozygous p.C169Y allele with Patient 4 but reveal a much more severe phenotype. Furthermore, all four missense mutants p.C65W, p.C105R, p.C169Y and p.N222S were found to be mistargeted to intracellular compartments and partly retained in the endoplasmic reticulum. Thus in consequence, all reported missense mutants of FRα are functionally incapable of binding to folate at the plasma membrane and ultimately cannot mediate the transport of folate compounds.

To date, our molecular studies do not consistently explain the phenotypic variations that we found among patients with cerebral folate transport deficiency. This indicates that additional factors contribute to the clinical phenotype of this neurodegenerative disorder. Possible genetic factors that could influence the severity of cerebral folate transport deficiency represent interindividual different expression profiles of other folate binding receptors or transporters. Interestingly, we found an age-dependent decrease in folate receptor β expression in human leukocytes (Steinfeld et al., 2009). It can be hypothesized that either prolonged expression of folate receptor β or accelerated myelination of the post-natal developing brain might favour a milder clinical phenotype. The time course of brain development and myelination might also depend on environmental factors such as nutrition and in particular on folate and choline intake. Although we surveyed only a small number of patients, the milder phenotype in cerebral folate transport deficiency only occurred in females and most interestingly, even among siblings. Additional investigations are required to understand the exact mechanism of cerebral folate transport and to fully comprehend the molecular pathology of its deficiency.

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Supplementary material

Supplementary material is available at Brain online.

References


