Dihydrofolate Reductase Deficiency Due to a Homozygous DHFR Mutation Causes Megaloblastic Anemia and Cerebral Folate Deficiency Leading to Severe Neurologic Disease

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The importance of intracellular folate metabolism is illustrated by the severity of symptoms and complications caused by inborn disorders of folate metabolism or by folate deficiency. We examined three children of healthy, distantly related parents presenting with megaloblastic anemia and cerebral folate deficiency causing neurologic disease with atypical childhood absence epilepsy. Genome-wide homozygosity mapping revealed a candidate region on chromosome 5 including the dihydrofolate reductase (DHFR) locus. DHFR sequencing revealed a homozygous DHFR mutation, c.458A>T (p.Asp153Val), in all siblings. The patients' folate profile in red blood cells (RBC), plasma, and cerebrospinal fluid (CSF), analyzed by liquid chromatography tandem mass spectrometry, was compatible with DHFR deficiency. DHFR activity and fluorescein-labeled methotrexate (FMTX) binding were severely reduced in EBV-immortalized lymphoblastoid cells of all patients. Heterozygous cells displayed intermediate DHFR activity and FMTX binding. RT-PCR of DHFR mRNA revealed no differences between wild-type and DHFR mutation-carrying cells, whereas protein expression was reduced in cells with the DHFR mutation. Treatment with folinic acid resulted in the resolution of hematological abnormalities, normalization of CSF folate levels, and improvement of neurological symptoms. In conclusion, the homozygous DHFR mutation p.Asp153Val causes DHFR deficiency and leads to a complex hematological and neurological disease that can be successfully treated with folinic acid. DHFR is necessary for maintaining sufficient CSF and RBC folate levels, even in the presence of adequate nutritional folate supply and normal plasma folate.

Various inherited and acquired disorders affecting folate uptake, transport, and metabolism have been described. Nutritional folate deficiency is one of the most common micronutrient deficiencies worldwide. Depending on the severity of folate deficiency, it is associated with megaloblastic anemia, neurologic and mental disorders, cardiovascular disease, embryonic defects (in particular neural tube defects), and, possibly, malignancies.1 Furthermore, the importance of intracellular folate metabolism for normal psychomotor and cognitive development is illustrated by the severity of symptoms in disorders of folate metabolism, such as methylenetetrahydrofolate reductase deficiency (MTHFR [MIM 236250]) or glutamate formiminotransferase deficiency (FTCD [MIM 229100]).2

Dihydrofolate reductase (DHFR [MIM 126060]) catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) and, at a lower rate, of folic acid (FA) to DHE. DHFR plays a key role in maintaining intracellular folate homeostasis and is an important target for cytostatic drugs. To date, three cases with initially suspected inherited DHFR deficiency have been reported.3,4 Later analyses revealed normal DHFR activity in one patient and the presence of transcobalamin II deficiency (TCN2 [MIM 275350]) as the underlying disorder in another of the reported patients.5,6 Follow-up for the third patient was not reported. A distinct genetic defect associated with DHFR deficiency has not been described.

We examined three children of healthy, distantly related parents of European descent (Figure 1A). Patient 1 (VII-1 in Figure 1) presented at age 11 without any clinical symptoms or findings. Peripheral-blood analysis revealed macrocytosis without anemia (Table 1). Electroencephalogram (EEG) at that time showed relatively slow 8–9/5 waves, particularly in the parieto-occipital region, and intermittently repetitive generalized high-amplitude delta waves but no epilepsy-specific potentials. Patient 2 (VII-2 in Figure 1) presented at age 5 with hemoglobin level at 56 g/l, mean corpuscular volume (MCV) at 117 fl, 3.3% reticulocytes, and serum lactate dehydrogenase activity at 2066 U/l without any congenital physical abnormality. Bone marrow examination revealed typical features of megaloblastic anemia. Serum folate, cobalamin, transcobalamin I and II binding capacity, and homocysteine, as well as urinary excretion of methylmalonic, orotic, and formimino glutamic acid, were normal. Treatment with hydroxycobalamin and FA (5 mg per day)

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normalized hemoglobin levels and MCV. At age 8, increasing learning difficulties developed and the patient presented with short episodes of involuntary blinking and winking, partially associated with impaired consciousness. EEG revealed atypical childhood absence epilepsy with eyelid myoclonia. A bone marrow smear still displayed mild megaloblastic changes despite an almost normal peripheral-blood analysis (Table 1).

Patient 3 (VII-3 in Figure 1) had a complicated febrile seizure at age 2. Blood analysis was normal apart from marked macrocytosis with MCV 109 fl. Physical examination did not reveal any pathological findings. Subsequent psychomotor and cognitive development was normal. At age 5, the girl presented with frequent episodes of impaired vision, blinking, and squinting associated with impaired consciousness and repetitive eyeball movements during sleep. EEG showed severe pathologic changes compatible with an atypical childhood absence epilepsy with eyelid myoclonia. Blood examination still showed isolated macrocytosis, whereas a bone marrow smear displayed megaloblastic changes (Table 1). The girl was treated with FA (5 mg per day) after consideration of the apparent effectiveness of FA in her brother, who was neurologically asymptomatic at that period.

After the occurrence of neurologic symptoms in patient 2 despite FA supplementation, 5-methyltetrahydrofolate (5-MTHF) and neurotransmitter concentrations in the cerebrospinal fluid (CSF) were determined for further assessment of the metabolic changes of the disease. 5-MTHF analysis was performed precisely as previously described. It revealed very low 5-MTHF in patient 1. In patients 2 and 3, CSF 5-MTHF was undetectable despite
preceding FA treatment (Table 1). Total red blood cell (RBC) folate was low in all patients. Initially, treatment with folinic acid (5-formyltetrahydrofolate; 5-FTHF) at 1 mg/kg per day resulted in CSF 5-MTHF normalization in patients 2 and 3; patient 1 was not reexamined. Total RBC folate increased in all patients (Table 1), associated with normalization of MCV and bone marrow morphology. Subsequently, patient 1 received 5-FTHF very irregularly. Three years later, he presented with focal epilepsy as his first clinical impairment. In patient 2, 5-FTHF treatment led to improved school performance, but persistent epilepsy. Patient 3 became transiently independent of anticonvulsant treatment and had no additional neurological symptoms. Later, irregular 5-FTHF treatment was associated with the recurrence of epileptic symptoms. The clinical and biochemical features of the patients were suggestive for an inherited disorder of folate transport or metabolism. Genomic sequencing, we excluded FOLR1 mutations known to cause congenital cerebral folate deficiency (CFD [MIM 613068]).

Consistent with a recessive inheritance model, we performed homozygosity mapping by using genome-wide SNP analysis performed on the Affymetrix GeneChip platform. This and all subsequent examinations were performed in accordance with the ethical standards of the ethics committee of the University of Ulm. Written informed consent was obtained from both parents. Genome-wide SNP analysis revealed a short overlap on the long arm of chromosome 2 and a second common region on the long arm of chromosome 5 (nucleotide 79613515–nucleotide 82033415, Human Genome Assembly, NCBI Human Genome Browser, build 36 [hg18]). This region, spanning 2.42 Mb, contains 28 known genes, including DHFR, the gene encoding for dihydrofolate reductase. Another gene locus known to be associated with folate transport or metabolism was not found within the overlapping homozygous regions. Genomic sequencing of DHFR exons and neighboring intronic nucleotides was performed after genomic DNA PCR reactions (for primer sequences, see Table S1 available online). Sequence analysis revealed a homozygous DHFR mutation, c.458A>T, in exon 5 (RefSeq NM_000791.3) leading to a p.Asp153Val substitution in all siblings, thus affecting an amino acid that, according to the Homologene database, is highly conserved (Figure 1B). The mutation has been deposited to the NCBI dbSNP. Both parents (VI-2 and VI-3 in Figure 1) are heterozygous for the mutation. The mutation was not detected in 120 control samples.

DHFR possesses an α/β fold with a central 8-stranded sheet, flanked by four α helices (Figure 1C). DHF and NADPH cofactor are bound in a deep cleft (active site cleft) that partitions the structure into two subdomains. Two prominent loops, denoted “Met20” and “F-G loop,” emerge from one subdomain and form a lid that buries and positions NADPH’s nicotinamide moiety in the cleft for hydride transfer. A variety of studies indicate that the dynamics of conformational states of these active site loops are critical for efficient catalysis.9 Asp153 is situated at the C-terminal end of the “F-G” loop, which connects
β sheets F and G. It forms a hydrogen bond with the backbone nitrogen of Gly155, thereby stabilizing a helical turn that is involved in anchoring sheet G and loops “F-G” as well as “G-H” to the remainder of the structure. Val153 will lack this hydrogen bond as well as the negative charge. The mutation likely affects fold, conformational stability, and dynamics of the “F-G” loop and its closely associated Met20 loop, resulting in an enzyme with reduced catalytic efficiency.

Assuming DHFR deficiency, we analyzed folate metabolites in RBCs and plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described. This assay distinguishes 5-MTHF, FA (sum of FA and DHA, which converts to FA during assay), nonmethylTHF, and unsubstituted THF. Total RBC folate was obtained by addition of the separate folate fractions or by a chemiluminescence immunoassay. Before treatment, patient 1 had low RBC folates despite normal plasma folates (Table 1). During 5-FTHF treatment, RBC 5-MTHF remained low in all patients. In addition, nonmethylTHF and FA were present. Appreciable concentrations of non-methyl folates in RBCs are normally observed only in individuals with the methylenetetrahydrofolate reductase (MTHFR [MIM 607093]) 677 TT but not the MTHFR 677 CC genotype. The latter genotype was found in all siblings. Therefore, the occurrence of non-methylTHF, which includes 5-FTHF, is probably caused by 5-FTHF treatment and not by DHFR deficiency. During 5-FTHF treatment, FA + DHF was remarkably high in RBCs, whereas THF remained low, which supports impaired DHF-to-THF conversion. DHF is the likely source for the measured FA + DHF, because no FA was used at the time and spontaneous formation of FA in vivo is believed to be virtually absent. Given that THF is the sole substrate for polyglutamate synthase and that polyglutamation is required for cellular storage of folates, the insufficient supply of THF from DHF may explain low total RBC folate levels.

Plasma and CSF homocysteine (Hcy) and methionine were normal. Apparently, in this situation, with normal nutritive folate supply and normal plasma folate, Hcy regulation by S-adenosylmethionine via MTHFR and cystathionine beta-synthase (CBS [MIM 618833]) pathways is sufficient to compensate for dysfunctional DHFR. This is in contrast to the Hcy increase observed in patients treated with methotrexate (MTX), which represents a high-affinity substrate for DHFR. However, because MTX is inhibiting not only DHFR but also other folate-related transport proteins and enzymes (including MTHFR, dihydropteridine reductase, etc.), its effect on Hcy is certainly of a more complex nature than being due to only DHFR inhibition. Neopterin, biotin, and neurotransmitter levels in the CSF and S-adenosyl-methionine and S-adenosylhomocysteine concentrations in the plasma and in RBC were all within the normal range. Leukocyte global DNA methylation, determined by LC-MS/MS and expressed as the methyl-cytosine percentage of total cytosine, was also normal and not influenced by 5-FTHF substitution (data not shown).

To examine DHFR function directly, we studied the formation of THF from DHF in EBV-immortalized lymphoblastoid cells and fibroblasts from patients and controls. Cells were incubated with NADPH and DHF, then stabilized with mercaptoethanol and frozen. Cells were lysed by freeze-thaw cycles. After centrifugation, the supernatant was acidified, an internal standard ([13C5]-5-methenylTHF) was added, and proteins were removed. THF concentration was determined by LC-MS/MS. DHFR activity in lymphoblastoid cells was severely reduced to less than 10% of control levels in all patient cells, whereas cells of the heterozygous mother (VI-3 in Figure 1) exhibited DHFR activity close to the lower limit of the control range (Figure 2A). A confirmation of these results in fibroblasts was not possible because under the assay conditions that were used, DHFR activity in fibroblasts was very low, even in wild-type cells (data not shown). Nevertheless, in the context of the genetic and biochemical data, the results for DHFR activity obtained in lymphoblastoid cells have to be regarded as representative for the disorder.

To assess binding of the substrate to the mutated DHFR, we performed a fluorescence-based assay, showing that binding of fluorescein-labeled methotrexate (FMTX) to DHFR is severely reduced in lymphoblastoid cells of all three patients and slightly decreased in cells of the heterozygous mother (Figure 2B).

To assess whether the reduced DHFR activity was caused by an altered mRNA and protein expression, we performed RT-PCR and immunoblot analyses (Figures 2C and 2D). In contrast to the DHFR mRNA expression, which did not differ between patients and controls (Figure 2C), protein expression was reduced in mutated cells as compared to wild-type cells. Immunoblotting revealed reduced intact DHFR in heterozygous (~70%) as well as in homozygous (~20%–50%) lymphoblastoid cells (Figure 2D). Protein reduction was less severe in mutated fibroblasts (heterozygous ~90%, homozygous 70%–80%). Probably, the mutation is responsible for the reduced protein expression by changing protein folding, binding to chaperones, or influencing the stability of other protein complexes involving DHFR.

However, both the DHFR activity and FMTX binding show an equally severe DHFR dysfunction, whereas the variable reduction of protein expression is only moderate (lymphoblasts) or mild (fibroblasts). Therefore, the interindividual protein variation probably reflects differences that are not intrinsic to DHFR but also depend on extrinsic factors that target DHFR directly or target its binding to stabilizing intracellular factors and, hence, contribute to the endogenous variability of cellular protein processing. With regard to the equally severe reduction of DHFR activity, the variation of the moderate protein reduction as a single factor cannot explain the phenotypic variability seen in patients with DHFR deficiency.
The causes underlying the different phenotype expression in individual patients remain obscure. Additional DHFR alterations that could possibly modify the effect of the DHFR p.Asp153Val mutation were excluded (data not shown). MTHFR activity in homozygous and heterozygous lymphoblastoid cells, as determined by LC-MS/MS, did not differ from that in wild-type cells (data not shown).

Biopterin and folate metabolism appear to overlap at several points, and previous in vitro studies demonstrated that human dihydropteridine reductase (DHPR [MIM 612676]) may function in THF metabolism.14,15 We therefore analyzed DHPR activity in dried blood samples16 from the three siblings and found that it did not differ from that of controls (data not shown). However, DHPR activity in the peripheral blood may not adequately reflect its activity in the brain.17

To summarize, we have shown that the homozygous DHFR mutation p.Asp153Val causes DHFR deficiency and leads to a complex neurological and hematological disease in the absence of any malformation, including neural tube defects. Apparently, intact intracellular folate metabolite recycling through DHFR is of central importance for maintaining sufficient CSF and RBC folate levels, even in the presence of adequate nutritional folate supply and normal plasma folate. CSF folate levels were comparable to those of patients with congenital or acquired cerebral folate deficiency who present in infancy or early childhood with severe neurological symptoms.8,18 It seems possible that in CFD patients, not only the low CSF folate but also other changes associated with folate receptor dysfunction contribute to the early disease manifestation and the more severe phenotype.

In conclusion, we have described patients with inherited DHFR deficiency due to homozygous DHFR mutation. This condition merits consideration in young patients who present with unexplained macrocytic anemia, moderately severe developmental disorders, and epilepsy.

Supplemental Data
Supplemental Data include one table and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources
The URLs for data presented herein are as follows:

Accession Numbers
The NCBI dbSNP accession number for the sequence reported in this paper is rs121913223.

References