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Identification of novel mutations in the proton-coupled folate transporter (PCFT-SLC46A1) associated with hereditary folate malabsorption

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Abstract

Hereditary folate malabsorption (HFM) is an autosomal recessive disorder, recently shown to be due to loss-of-function mutations of the proton-coupled folate transporter (PCFT-SLC46A1), resulting in systemic and central nervous system folate deficiency. Data is emerging on the spectrum of PCFT mutations associated with this disorder. In this report, novel mutations are described in three subjects with HFM: A335D/N68Kfs (c.1004C>A/ c.204-205delCC), a compound heterozygous mutation, and two homozygous PCFT mutations, G338R (c.1012G>C) and E9Gfs (c.17-18insC). Functional assessment of A335D and G338R PCFT mutants transfected into folate transporter-deficient HeLa R1-11 cells indicated a complete loss of transport activity. There were neurological deficiencies in two of the families reported; in particular, late-onset seizures. The importance of early diagnosis and treatment to achieve *physiological* cerebrospinal fluid folate levels is emphasized.

Keywords

HFM; hereditary folate malabsorption; PCFT; proton-coupled folate transporter; HCP1; PCFT/HCP1; heme carrier protein1; folates; proton-coupled transport; intestinal folate transport

Introduction

Hereditary Folate Malabsorption (HFM) is an autosomal recessive disorder characterized by impaired intestinal folate absorption and impaired transport of folates into the central nervous system [4,9]. Recently, this laboratory cloned the proton-coupled folate transporter (PCFT-SLC46A1) and established that mutations in this gene are the molecular basis for

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HFM [14,23]. Since then additional subjects with the clinical syndrome of HFM, and loss-of-function *pcft* mutations in this gene, have been reported by this and other laboratories [1,2,7,8,10,11,17].

The *pcft* gene consists of 5 exons spanning ~6.5Kb of genomic DNA. The PCFT protein consists of 459 amino acids; its topology has been established by the substituted cysteine accessibility method. PCFT encompasses twelve transmembrane domains with N- and C-termini directed to the cytoplasm [15,19,25]. There is a disulfide bond between the C66 and C298 residues in the first and fourth extracellular loops, respectively, but this is not required for function [25].

In the initial report, two siblings of Puerto Rican ancestry with HFM had a mutation in the splice acceptor of exon 2 at the intron2/exon 3 boundary resulting in the skipping of exon 3 and a protein that does not traffic to the cell membrane [14]. Since then, another patient from Puerto Rico with this mutation has been reported [2]. In other subjects with HFM, a variety of mutations have been detected at different locations: (i) R113S and R113C in the 1st intracellular loop between 2nd and 3rd TMDs [7,23], (ii) P425R at the junction of the 6th extracellular loop and 12th TMD [23]. (iii) R376W and R376Q in the 10th TMD [8,23], (iv) G147R, D156Y in the 4th TMD [17,23]; and (v) S318R in the 8thTMD [23]. (vi) Several mutations in the first extracellular loop have been identified that lead to frameshifts, truncated and nonsense proteins [1,10,11,23]. In the current paper, four loss-of-function *pcft* mutations are reported in three subjects: A335D and N68Kfs, a compound heterozygous mutation in one subject, and homozygous mutations, E9Gfs and G338R, in two other subjects.

Methods

Identification of novel mutations in patients with HFM

These studies were approved by the Albert Einstein College of Medicine Institutional Review Board. After informed consent was obtained from the patients and their family members, peripheral blood was collected. IRB-approved consent forms translated into French were used with the Tunisian subjects. Genomic DNA was isolated at the Albert Einstein College of Medicine DNA Isolation and Cell Expansion Core. Each of 5 exons of the *pcft* gene along with the flanking regions was amplified by PCR with primers as reported previously [14]. PCR products were analyzed on an agarose gel, purified by a gel purification kit (GE Healthcare, Buckinghamshire, HP7 9NA, UK), and sequenced at the Albert Einstein College of Medicine Cancer Center Genomics Shared Resource.

Chemicals—Methotrexate (MTX-disodium salt, [3', 5', 7-³H](N)] was obtained from Moravsek Biochemicals Inc (Brea, CA) and purified by liquid chromatography before use as previously described [21].

Construction of mutant plasmids by site-directed mutagenesis—Mutations were introduced into *pcft* cDNA with the Quick change II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations in the plasmid constructs were verified by DNA sequence analysis. A PCFT pcDNA3.1(+) expression vector, which encodes HA-tagged PCFT at the C-terminus, was used as the template for all site-directed mutants.

Cell lines, cell culture conditions and transient transfection—HeLa-R1-11 cells are a stable subclone of the HeLa R1 cell line that lacks both RFC and PCFT expression [24], the former due to a genetic deletion [22], the latter due to methylation of the promoter and loss of gene copies [3]. R1-11 cells are maintained in RPMI-1640 medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml

streptomycin at 37°C in a humidified atmosphere of 5% CO₂. For transport studies, R1-11 cells (3.5×10^5 /vial) were seeded into 17-mm liquid scintillation vials. After 48 hours, the various constructs (0.8 µg/vial), were transiently transfected into the cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA). The cells were processed for transport assays two days later.

Membrane transport analyses

A technique for rapid measurement of [³H]MTX influx was employed, (MTX is a stable, inexpensive surrogate for physiological folates) [16]. R1-11 transfectants were washed twice with 2 ml of HBS buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM dextrose at pH 7.4) and incubated in the same buffer in a water bath (37°C) for 20 min. The buffer was then aspirated following which transport buffer, 500 µl of MBS (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM dextrose at pH 5.5) containing 0.5 µM [³H]MTX was added. [³H]MTX uptake was halted after 1 min by the addition of 10 volumes of ice-cold HBS buffer (pH 7.4), an interval over which uptake was unidirectional. Cells were then washed 3 times with 5 ml of ice-cold HBS buffer, following which 500 µl of 0.2M NaOH was added and cells digested by incubation for 30-45 min at 65°C. A portion of the hydrolysate (400 µl) was then analyzed on a liquid scintillation spectrometer; the protein content of another portion of hydrolysate (10 µl) was determined using the Pierce kit (Thermo Scientific, Rockford, IL). Influx is expressed as pmoles of [³H]MTX per mg of protein per min and data is reported as mean ± SEM from at least three independent experiments performed on different days.

Results

Clinical findings

Subject P1—This male subject (P1), currently age 6 years, was born to non-consanguineous English parents whose heritage is traced to both sets of English grandparents. There was no pertinent family history. At age 2 months, the child developed *Pneumocystis jiroveci* pneumonia. His hemoglobin (Hb) was 4 g/dL; plasma folate was undetectable. Serum IgG was normal but IgA and IgM levels were low. The bone marrow biopsy showed megaloblastic changes. He received IV leucovorin and cotrimoxazole, and the pneumonia resolved. The patient was discharged on oral folic acid, 1.5 mg/day. At age 5 months, Hb was 12.4 g/dL, mean corpuscular volume (MCV) 88.5 fL, white blood cell (WBC) 9.8×10^9 /L. The serum folate was 2.6 nM. At age 10 months, the serum folate was 11.9 nM and red blood cell (RBC) folate was 97 ng/mL (nl: 150-650 ng/mL). A magnetic resonance image of the head was normal; a lumbar puncture was unsuccessful. The folic acid dose was increased to 2.5 then 5 mg/day. At age 16 months the patient had not as yet walked, had a resting tremor, hyper-reflexia, jerky movements, and proximal muscle wasting. Hb was 11.5 g/dL, MCV 86, WBC 7.6×10^9 /L, serum folate 13.7 nM, RBC folate 91 ng/mL. Blood homocysteine was 27 µM (nl: 5-15 µM). The folic acid dose was increased to 10 then 15 mg/day. At age 18 months, the serum folate had increased to 18.9 nM and the RBC folate level was 120 ng/mL. However, the cerebrospinal fluid (CSF) folate level was undetectable. The patient was started on IM leucovorin (5-formylTHF) 5 mg/day. One month later, his neurological status improved; he was able to stand without support. He continued to improve neurologically except for some difficulty in fine motor skills. The leucovorin dose was increased and, at 12 mg IM/d, the CSF folate level was 30 nM, (normal for this age - 70-90 nM; see Discussion). At age 3 years 9 months, a magnetic resonance image of the head showed a slight delay in myelination. The patient steadily improved neurologically but with continued mild difficulty in fine motor skills and reading but good math skills. At age 5 years 4 months the magnetic resonance image of the head was normal. A few months later, the patient began to experience episodes in which he appeared to have a

loss of vision associated with contraction of his pupils that increased in time to a frequency of ~30 times each day. An EEG was consistent with occipital seizures. He was begun on antiseizure medications. The patient currently receives 12 mg leucovorin IM/day with 15 mg/day PO. The CSF folate trough level was 14 nM in October 2009 and 17 nM October 2010.

Subject P2—A male subject (P2), now age 20, included in an earlier report of a Tunisian family in which, of eight children, five died within the first few months of life with diarrhea, pallor and glossitis. The three surviving children, two boys and one girl, were reported to have the same abnormalities [5]. Studies on the female sibling (now age 21) at age 3 months revealed a severe macrocytic anemia with marked folate deficiency and negligible CSF folate. Absorption of an oral load of folic acid was abnormal. The patient was treated with IM leucovorin (50 mg) which was said to achieve “acceptable” CSF folate levels. However, leucovorin could not be administered regularly. The patient developed seizures at age 4 and her IQ was noted to be low. Two brothers, one of whom (P2) is the subject of the current report, were said to have similar hematological problems that resolved in infancy with intermittent IM 5-formylTHF. At ages 9 and 10 years, respectively, they were noted to have low IQs and poor performance at school. Their cognitive and neurological status has remained stable since that time.

Subject P3—This Tunisian male (P3) born from consanguineous parents (2nd degree) and currently age 8, developed macrocytic anemia with low serum folate at age 2.5 months. One sibling died at birth, another died at 7 months due to pancytopenia, immune deficiency and severe respiratory distress. The physician had previously treated patient 2 so that the diagnosis in this case was made rapidly with initiation of IM leucovorin (10 mg/day) that was subsequently decreased to weekly, then biweekly, with PO leucovorin at 50 mg/day. The anemia rapidly corrected along with the peripheral and axial hypertonia noted initially. An EEG and head computed tomography were normal; however, CSF folate remained low. At age 19 months, CSF folate continued to be barely detectable; at age 3 years 8 months, CSF folate was undetectable. The patient continues on leucovorin.

Identification of novel mutations in subjects with HFM

P1 had compound heterozygous *pcft* mutations (Figure 1, upper panel). The mutation inherited from the heterozygous father, a c.1004C>A (NM_080669) nucleotide substitution, was located in exon 2 and led to an Ala to Asp substitution at the 335 residue (A335D) located in the 9th TMD (Figure 2) (NP 54200.2). The mother, also heterozygous, contributed an allele with deletion of 2 nucleotides, CC, at positions 204 and 205 (c.204-205delCC) in exon 1 that caused a frameshift starting at position N68 with early termination of translation. The maternal allele was traced back to the maternal grandmother; the paternal allele was traced back to the paternal grandmother (Figure 1, lower panel). P2 had a homozygous C insertion in an all-C region (bases #18-23) causing an early frameshift starting at E9 and, a truncated nonsense protein. P3 had a homozygous G→C mutation at position 1012 (c.1012G>C). This resulted in a G338R substitution also within the 9th TMD. The A335 and G338 residues are fully conserved (to and including zebrafish) among species. These novel mutations were not seen in the NCBI SNP database.

Functional assessment of A335D and G338R PCFT mutants

A335D (c.1004C>A; P1) and G338R (c.1012G>C; P3) PCFT mutants were generated by site-directed mutagenesis as described above and transfected into R1-11 cells. [³H]MTX influx was assessed at pH 5.5 and 37°C over 1 min. As Figure 3 indicates, A335D and G338 PCFT mutants were inactive with uptake no different from cells transfected with empty

vector (mock). The PCFT mutation detected in P2 resulted in a very early frame-shift and nonsense protein obviating the need for functional evaluation.

Discussion

This paper adds three new PCFT mutations to the evolving knowledge of the molecular changes in the PCFT protein associated with HFM. A335D (c.1004C>A), in the 9th TMD, was detected in one allele of an English patient traced back to an English grandparent. A different mutation was detected in the second allele, N68Kfs (c.204-205delCC), also traced back to an English grandparent (the great-grandparents were also of English heritage). It is of interest that the latter mutation was recently detected in two siblings from a family in Turkey (in this case homozygous) [1]. Two families from Tunisia are reported both cared for by the same physician. One of these families, in which the disorder appeared to be prevalent among eight siblings, was described prior to the cloning of PCFT [5]. A male sibling from this family is reported with a homozygous mutation due to a C insertion in an all-C region (c.18_23) resulting in a frameshift, E9Gfs (c.17-18insC). A different homozygous mutation was detected in the second subject from a Tunisian family, c.1012G>C resulting in a G338R substitution. The current availability of a genetic test for this disorder now makes it possible to confirm the diagnosis of HFM, and identify carriers, in these and other families particularly when the mutation is known and the test should be relatively inexpensive.

Immune deficiency due to hypoglobulinemia is an important complication of HFM often manifested by *Pneumocystis jiroveci* pneumonia, as was documented for P1 [4,9,18,23]. The immune deficiency is rapidly reversed with systemic folate repletion and correction of the hematopoietic manifestations of HFM. Neurological disorders, in particular seizures, also frequently accompany HFM [4,8,9,17]. It is unclear as to why some patients develop seizures and others do not, why the onset of seizures can be delayed, as in the first subject (P1) and a sibling of the second subject (P2), and whether this is related solely to the adequacy of treatment. CSF folate levels are very high during infancy and decrease only slowly during the first decade. CSF folate levels for the first year are 100-150 nM, decreasing to ~70-90 nM by age 5 and remaining in the 60-90 nM range well into the teens [12,13,20]. Hence, the endpoint of treatment is to achieve and maintain normal blood, and red blood cell, folate levels and CSF folate levels appropriate to the age of the patient.

Because of the level of CSF folate required for the treatment of HFM, intramuscular dosing should be the most efficacious mode of administration. Leucovorin (racemic 5-formylTHF), the folate most frequently utilized, is rapidly metabolized to the physiological 5-methyltetrahydrofolate. Isovorin (calcium levofolinate – Wyeth), the active isomer of 5-formylTHF, is available for parenteral administration. The oral formulation of 5-methyltetrahydrofolate (Metafolin – Merck) is available but the strength is insufficient for treatment of this disorder. Folic acid should not be used since it binds so tightly to folate receptors that it may impair the transport of 5-methylTHF across the blood-choroid plexus-CSF barrier [6].

The first exon, particularly nucleotides encoding the first extracellular loop between the first and second transmembrane domains, has a high GC content, 65.8% (75% between residues 63 and 70) and is the most frequent site of PCFT mutations in subjects with HFM. Beyond the subjects described above, three other mutations have been reported in the first exon. These include a stop codon (c. 197 GC>AA; C66X) and three frame-shifts (G65Afs, C66Lfs and N68Kfs) [1,10,23]. All the other mutations associated with HFM, aside from the one resulting in the formation of a splice variant [1,10,23], have been within or at the junction of TMDs [8,17,23]. Two residues have been the sites of two different mutations in different patients: R113S/C and R376W/Q [7,8,23].

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Reference List

- Atabay B, Turker M, Ozer EA, Mahadeo K, Diop-Bove N, Goldman ID. Mutation of the Proton-Coupled Folate Transporter Gene (PCFT-SLC46A1) in Turkish Siblings With Hereditary Folate Malabsorption. *Pediatr Hematol Oncol*. 2010; 27:614–619. [PubMed: 20795774]
- Borzutzky A, Crompton B, Bergmann AK, Giliani S, Baxi S, Martin M, Neufeld EJ, Notarangelo LD. Reversible Severe Combined Immunodeficiency Phenotype Secondary to a Mutation of the Proton-Coupled Folate Transporter. *Clin Immunol*. 2009; 133:287–294. [PubMed: 19740703]
- Diop-Bove NK, Wu J, Zhao R, Locker J, Goldman ID. Hypermethylation of the Human Proton-Coupled Folate Transporter (SLC46A1) Minimal Transcriptional Regulatory Region in an Antifolate-Resistant HeLa Cell Line. *Molecular Cancer Therapeutics*. 2009; 8:2424–2431. [PubMed: 19671745]
- Geller J, Kronn D, Jayabose S, Sandoval C. Hereditary Folate Malabsorption: Family Report and Review of the Literature. *Medicine (Baltimore)*. 2002; 81:51–68. [PubMed: 11807405]
- Jebnoun S, Kacem S, Mokrani CH, Chabchoub A, Khrouf N, Zittoun J. A Family Study of Congenital Malabsorption of Folate. *J Inher Metab Dis*. 2001; 24:749–750. [PubMed: 11804211]
- Kamen BA, Smith AK. A Review of Folate Receptor Alpha Cycling and 5-Methyltetrahydrofolate Accumulation With an Emphasis on Cell Models in Vitro. *Adv Drug Deliv Rev*. 2004; 56:1085–1097. [PubMed: 15094208]
- Lasry I, Berman B, Straussberg R, Sofer Y, Bessler H, Sharkia M, Glaser F, Jansen G, Drori S, Assaraf YG. A Novel Loss of Function Mutation in the Proton-Coupled Folate Transporter From a Patient With Hereditary Folate Malabsorption Reveals That Arg 113 Is Crucial for Function. *Blood*. 2008; 112:2055–2061. [PubMed: 18559978]
- Mahadeo K, Diop-Bove N, Shin D, Unal E, Teo J, Zhao R, Chang MH, Fulterer A, Romero MF, Goldman ID. Properties of the Arg376 Residue of the Proton-Coupled Folate Transporter (PCFT-SLC46A1) and a Glutamine Mutant Causing Hereditary Folate Malabsorption. *Am J Physiol Cell Physiol*. 2010; 299:C1153–C1161. [PubMed: 20686069]
- Mahadeo, KM.; Min, SH.; Diop-Bove, N.; Kronn, D.; Goldman, ID. Hereditary Folate Malabsorption, in *GeneReviews* [Internet]. Pagon, RA.; Bird, TC.; Dolan, CR.; Stephens, K., editors. University of Washington, Seattle; Seattle,WA: 2010.
- Meyer E, Kurian MA, Pasha S, Trembath RC, Cole T, Maher ER. A Novel PCFT Gene Mutation (p.Cys66LeufsX99) Causing Hereditary Folate Malabsorption. *Mol Genet Metab*. 2010; 99:325–328. [PubMed: 20005757]
- Min SH, OH SY, Karp GI, Poncz M, Zhao R, Goldman ID. The Clinical Course and Genetic Defect in the PCFT in a 27-Year-Old Woman With Hereditary Folate Malabsorption. *J Pediatr*. 2008; 153:435–437. [PubMed: 18718264]
- Opladen T, Ramaekers VT, Heimann G, Blau N. Analysis of 5-Methyltetrahydrofolate in Serum of Healthy Children. *Mol Genet Metab*. 2006; 87:61–65. [PubMed: 16310391]
- Ormazabal A, Garcia-Cazorla A, Perez-Duenas B, Gonzalez V, Fernandez-Alvarez E, Pineda M, Campistol J, Artuch R. Determination of 5-Methyltetrahydrofolate in Cerebrospinal Fluid of Paediatric Patients: Reference Values for a Paediatric Population. *Clin Chim Acta*. 2006; 371:159–162. [PubMed: 16624264]
- Qiu A, Jansen M, Sakaris A, Min SH, Chattopadhyay S, Tsai E, Sandoval C, Zhao R, Akabas MH, Goldman ID. Identification of an Intestinal Folate Transporter and the Molecular Basis for Hereditary Folate Malabsorption. *Cell*. 2006; 127:917–928. [PubMed: 17129779]
- Qiu A, Min SH, Jansen M, Malhotra U, Tsai E, Cabelof DC, Matherly LH, Zhao R, Akabas MH, Goldman ID. Rodent Intestinal Folate Transporters (SLC46A1): Secondary Structure, Functional

- Properties, and Response to Dietary Folate Restriction. *Am J Physiol Cell Physiol*. 2007; 293:C1669–C1678. [PubMed: 17898134]
16. Sharif KA, Goldman ID. Rapid Determination of Membrane Transport Parameters in Adherent Cells. *BioTechniques*. 2000; 28:926–8. 930, 932. [PubMed: 10818699]
 17. Shin DS, Min SH, Russell L, Zhao R, Fiser A, Goldman ID. Functional Roles of Aspartate Residues of the Proton-Coupled Folate Transporter (PCFT; SLC46A1); a D156Y Mutation Causing Hereditary Folate Malabsorption. *Blood*. 2010; 116:5162–5169. [PubMed: 20805364]
 18. Unal ES, Zhao R, Goldman ID. Role of the Glutamate 185 Residue in Proton Translocation Mediated by the Proton-Coupled Folate Transporter SLC46A1. *Am J Physiol Cell Physiol*. 2009; 297:C66–C74. [PubMed: 19403800]
 19. Unal ES, Zhao R, Qiu A, Goldman ID. N-Linked Glycosylation and Its Impact on the Electrophoretic Mobility and Function of the Human Proton-Coupled Folate Transporter (HsPCFT). *Biochim Biophys Acta*. 2008; 1178:1407–1414. [PubMed: 18405659]
 20. Verbeek MM, Blom AM, Wevers RA, Lagerwerf AJ, van de GJ, Willemsen MA. Technical and Biochemical Factors Affecting Cerebrospinal Fluid 5-MTHF, Biopterin and Neopterin Concentrations. *Mol Genet Metab*. 2008; 95:127–132. [PubMed: 18722797]
 21. Zhao R, Babani S, Gao F, Liu L, Goldman ID. The Mechanism of Transport of the Multitargeted Antifolate, MTA-LY231514, and Its Cross Resistance Pattern in Cell With Impaired Transport of Methotrexate. *Clin Cancer Res*. 2000; 6:3687–3695. [PubMed: 10999762]
 22. Zhao R, Gao F, Hanscom M, Goldman ID. A Prominent Low-PH Methotrexate Transport Activity in Human Solid Tumor Cells: Contribution to the Preservation of Methotrexate Pharmacological Activity in HeLa Cells Lacking the Reduced Folate Carrier. *Clin Cancer Res*. 2004; 10:718–727. [PubMed: 14760095]
 23. Zhao R, Min SH, Qiu A, Sakaris A, Goldberg GL, Sandoval C, Malatack JJ, Rosenblatt DS, Goldman ID. The Spectrum of Mutations in the PCFT Gene, Coding for an Intestinal Folate Transporter, That Are the Basis for Hereditary Folate Malabsorption. *Blood*. 2007; 110:1147–1152. [PubMed: 17446347]
 24. Zhao R, Qiu A, Tsai E, Jansen M, Akabas MH, Goldman ID. The Proton-Coupled Folate Transporter (PCFT): Impact on Pemetrexed Transport and on Antifolate Activities As Compared to the Reduced Folate Carrier. *Mol Pharmacol*. 2008; 74:854–862. [PubMed: 18524888]
 25. Zhao R, Unal ES, Shin DS, Goldman ID. Membrane Topological Analysis of the Proton-Coupled Folate Transporter (PCFT-SLC46A1) by the Substituted Cysteine Accessibility Method. *Biochemistry*. 2010; 49:2925–2931. [PubMed: 20225891]

Abbreviations

PCFT	proton-coupled folate transport (SLC46A1)
5-formylTHF	5-formyltetrahydrofolate (leucovorin)
HFM	hereditary folate malabsorption
TMD	transmembrane domain
CSF	cerebrospinal fluid

Figure 1. Chromatograms showing mutations in the *pcft* gene in three subjects with HFM; pedigree of subject P1

Upper panel: Subject P1 had compound heterozygous mutations. Subjects P2 and P3 had homozygous mutations. Lower panel: P1's pedigree traces heterozygous mutations, c. 1004C>A (A335D) and c.204-205delCC (N68Kfs), to the subject's paternal and maternal grandmothers, respectively.

Figure 2. PCFT topology and the location of mutations associated with hereditary folate malabsorption

The closed circles indicate the mutated residues described in this paper along with other reported mutations.

Figure 3. Functional assessment of A335D and G338R PCFT mutants

[³H]MTX influx was performed at a concentration of 0.5 μ M at pH 5.5 over 1 min with R1-11 cells transfected with A335D (one allele of P1) and G338R (P3) PCFT mutant plasmids along with wild-type PCFT and empty vector (mock).