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ARTICLE

Adenosine Kinase Deficiency Disrupts the Methionine Cycle and Causes Hypermethioninemia, Encephalopathy, and Abnormal Liver Function

Magnus K. Bjursell,[1,2] Henk J. Blom,[3] Jordi Asin Cayuela,[4] Martin L. Engvall,[2,5,6] Nicole Lesko,[6,7] Shanti Balasubramaniam,[8] Göran Brandberg,[9] Maria Halldin,[10] Maria Falkenberg,[11] Cornelis Jakobs,[3] Desiree Smith,[3] Eduard Struys,[3] Ulrika von Döbeln,[6,7] Claes M. Gustafsson,[11] Joakim Lundeberg,[12] and Anna Wedell[1,2,6,*]

Four inborn errors of metabolism (IEMs) are known to cause hypermethioninemia by directly interfering with the methionine cycle. Hypermethioninemia is occasionally discovered incidentally, but it is often disregarded as an unspecific finding, particularly if liver disease is involved. In many individuals the hypermethioninemia resolves without further deterioration, but it can also represent an early sign of a severe, progressive neurodevelopmental disorder. Further investigation of unclear hypermethioninemia is therefore important. We studied two siblings affected by severe developmental delay and liver dysfunction. Biochemical analysis revealed increased plasma levels of methionine, S-adenosylmethionine (AdoMet), and S-adenosylhomocysteine (AdoHcy) but normal or mildly elevated homocysteine (Hcy) levels, indicating a block in the methionine cycle. We excluded S-adenosylhomocysteine hydrolase (SAHH) deficiency, which causes a similar biochemical phenotype, by using genetic and biochemical techniques and hypothesized that there was a functional block in the SAHH enzyme as a result of a recessive mutation in a different gene. Using exome sequencing, we identified a homozygous c.902C>A (p.Ala301Glu) missense mutation in the adenosine kinase gene (*ADK*), the function of which fits perfectly with this hypothesis. Increased urinary adenosine excretion confirmed ADK deficiency in the siblings. Four additional individuals from two unrelated families with a similar presentation were identified and shown to have a homozygous c.653A>C (p.Asp218Ala) and c.38G>A (p.Gly13Glu) mutation, respectively, in the same gene. All three missense mutations were deleterious, as shown by activity measurements on recombinant enzymes. ADK deficiency is a previously undescribed, severe IEM shedding light on a functional link between the methionine cycle and adenosine metabolism.

Introduction

Hypermethioninemia can indicate a severe underlying genetic disorder, but it can also result from a benign inborn error of metabolism (IEM) or have a nongenetic cause. Four IEMs are known to cause hypermethioninemia by directly interfering with methionine or homocysteine metabolism. Methionine (Met) is normally converted to homocysteine (Hcy) in three enzymatic steps (Figure 1). Methionine adenosyltransferase (MAT) I/III deficiency (MIM 250850) affects the first step, the transfer of an adenosyl group from ATP to Met, which normally generates S-adenosylmethionine (AdoMet).[1] MAT I/III deficiency is clinically benign in most cases. Glycine-N-methyltransferase (GNMT) is one of about 60 methyltransferases that have been identified in mammals and that rely on AdoMet for methyl group donation. GNMT deficiency (MIM 606664) affects the quantitatively important methylation of glycine to form sarcosine[2] and thereby results in AdoMet accumulation. Affected individuals reported so far have shown discrete clinical symptoms, mainly from the liver. S-adenosylhomocysteine hydrolase (SAHH) deficiency (MIM 613752) affects the normal hydrolysis of S-adenosylhomocysteine (AdoHcy) to Hcy and adenosine and causes a severe clinical syndrome including myopathy, liver dysfunction, and grossly abnormal psychomotor development.[3] Finally, cystathionine beta-synthase (CBS) deficiency (MIM 236200) disrupts the first step of the transsulfuration pathway that normally degrades Hcy, and this disruption causes homocysteinuria.[4] The hypermethioninemia in homocysteinuria due to CBS deficiency is believed to result from remethylation of the excessive levels of Hcy into Met.[5] CBS deficiency is associated with dislocation of the optic lenses, early thromboembolic events, skeletal abnormalities, and mental retardation. In addition, elevated Met levels can be seen in fumarylacetoacetate hydrolase (FAH) deficiency (tyrosinemia type 1 [MIM 276700]) and citrullinemia type 2 (CTLN2) or citrin deficiency (MIM 605814). In FAH deficiency, elevated levels

[1]Department of Molecular Medicine and Surgery, Science for Life Laboratory, Karolinska Institutet, 17176 Stockholm, Sweden; [2]Center for Molecular Medicine, Karolinska Institutet, 17176 Stockholm, Sweden; [3]Department of Clinical Chemistry, VU University Medical Center, 1081 HV Amsterdam, The Netherlands; [4]Department of Clinical Chemistry, Sahlgrenska University Hospital, 41345 Gothenburg, Sweden; [5]Department of Molecular Medicine and Surgery, Karolinska Institutet, 17176 Stockholm, Sweden; [6]Centre for Inherited Metabolic Diseases, Karolinska University Hospital, 14186 Stockholm, Sweden; [7]Department of Laboratory Medicine, Karolinska Institutet, 14183 Stockholm, Sweden; [8]Genetics Department, Kuala Lumpur Hospital, Kuala Lumpur 50586, Malaysia; [9]Department of Pediatrics, Falu lasarett, 79182 Falun, Sweden; [10]Department of Women's and Children's Health, Akademiska University Hospital, 75105 Uppsala, Sweden; [11]Institute of Biomedicine, University of Gothenburg, 40530 Gothenburg, Sweden; [12]Science for Life Laboratory, Royal Institute of Technology, School of Biotechnology, 17121 Solna, Sweden
*Correspondence: anna.wedell@ki.se
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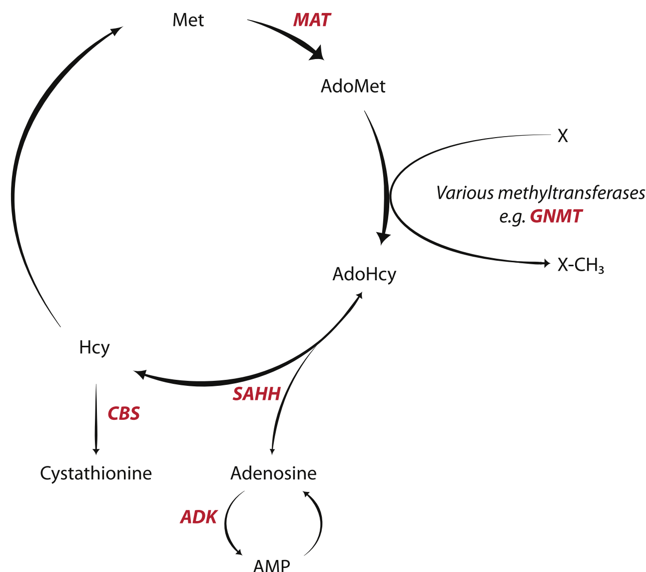


Figure 1. The Methionine Cycle and Its Relationship with the Adenosine/AMP Futile Cycle
Met is converted through AdoMet and AdoHcy to Hcy, which is subsequently remethylated back to Met. AdoMet functions as a methyl-group donor in a wide range of transmethylation reactions. The thermodynamics of the SAHH reaction favors condensation of adenosine and Hcy to produce AdoHcy; physiologically, AdoHcy is hydrolyzed when adenosine and Hcy are removed, and increased levels of adenosine therefore cause reversal of the reaction.
Adenosine is phosphorylated by ADK to AMP, which can be dephosphorylated back to adenosine by 5′ nucleotidase. This so-called futile cycle, which is disrupted in adenosine kinase deficiency, is considered an important regulator of adenosine and adenine nucleotide levels. MAT, GNMT, SAHH, and CBS are deficient in previously known IEMs that directly interfere with the methionine cycle and result in hypermethioninemia.
Abbreviations are as follows: ADK, adenosine kinase; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; AMP, adenine mononucleotide; CBS, cystathionine beta-synthase; GNMT, glycine N-methyl transferase; Hcy, homocysteine; MAT, methionine adenosyltransferase; Met, methionine; and SAHH, S-adenosylhomocysteine hydrolase.

of fumarylacetate are presumed to inhibit MAT III, whereas in citrin deficiency, the mechanism in unknown. Hypermethioninemia can also be seen in liver disease and sometimes in low-birth-weight and premature children, and in these cases it is often transient.[5] The underlying mechanisms for these forms are unclear. Met is included in many neonatal screening programs that aim to uncover CBS deficiency, and methods of establishing and discriminating between the different genetic causes of hypermethioninemia are essential because of the specific treatment modalities that are available for the different conditions and their drastically different prognoses.

Exome sequencing, i.e., targeted resequencing of all protein-coding sequences in our genome, is revolutionizing diagnostics in medical genetics. IEMs are ideal targets because their biochemical nature provides a functional context facilitating validation of encountered genetic variants. We applied exome sequencing to two siblings who were affected by severe developmental delay and liver dysfunction and who also displayed hypermethioninemia. Biochemical analysis revealed elevated plasma levels of Met, AdoMet, and AdoHcy but normal or mildly elevated levels of Hcy (tHcy). Deficiency of SAHH, caused by mutations in *AHCY* (MIM 180960), is known to cause a similar biochemical phenotype.[3] We excluded this diagnosis by using a combination of genetic and biochemical techniques and hypothesized that the siblings had a functional block in this enzymatic step as a result of a recessive mutation in a different gene. Exome sequencing revealed a homozygous mutation in *ADK* (MIM 102750), the function of which fits perfectly with this hypothesis. Two additional unrelated pairs of siblings with a similar biochemical phenotype were subsequently investigated and were found to have homozygous mutations in the same gene.

Subjects and Methods

Clinical Description

We studied two Swedish siblings, a boy born in 1987 and a girl born in 1996, with persistent isolated hypermethioninemia, severe developmental delay, and mild liver dysfunction. The parents were reportedly nonconsanguineous and healthy. An older male sibling had previous surgery due to congenital anal atresia but was otherwise healthy.

In the neonatal period both individuals were evaluated for failure to thrive, and the older brother was also investigated for prolonged conjugated hyperbilirubinemia. At a few months of age, hypotonia, profound psychomotor delay, and liver dysfunction were diagnosed in both affected children. The children later developed dysmorphic features with macrocephalus, frontal bossing, hypertelorism, and slender hands and feet. Epileptic seizures with early debut (before age 3) were present in both cases. The clinical course was thereafter similar in both individuals and was characterized by global psychomotor delay with sparse or absent language, slowly progressive muscular weakness, and muscle wasting. Epilepsy consisting of both partial and generalized seizures continued in the boy until adulthood and tended to be poorly controlled by antiepileptic therapy. EEG investigations showed slow background activity with diffuse multifocal discharges in some instances and abundant spike slow-wave complexes of high amplitude resembling Lennox-Gastaut in others. The girl died during sleep at the age of 10 years and 9 months.

Laboratory investigations showed persistent isolated hypermethioninemia in both children; maximum plasma Met values were 455 and 886 μmol/liter, respectively (reference range: 15–35 μmol/liter). Further investigations showed that total plasma Hcy (tHcy) levels were normal or slightly increased, thereby excluding homocystinuria due to CBS deficiency. Both AdoMet and AdoHcy were elevated (Table 1), making deficiency of MAT I/III unlikely. SAHH deficiency was ruled out by sequence analysis of *AHCY* and determination of enzyme activity in fibroblasts from the affected individuals. Tests showed abnormal liver function, including a moderate increase of liver transaminases

Table 1. Clinical and Biochemical Characteristics of the Three Families with ADK Deficiency

	Swedish Family		Malaysian Family 1		Malaysian Family 2	
	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6
Sex	male	female	male	male	male	female
Birth year	1987	1996	1992	1999	2000	2003
Met, plasma (μmol/liter)	455 (ref. 15–35)	886	800 (ref. 13–45)	550	800	600
Met, CSF (μmol/liter)	44 (ref. < 5)	41	n.a.	n.a.	n.a.	n.a.
AdoMet, plasma (nmol/liter)	677 (ref. 55–116)	496	153	335	303	656
AdoMet, CSF (nmol/liter)	264 (ref. 127–259)	291	n.a.	n.a.	n.a.	n.a.
AdoHcy, plasma (nmol/liter)	122 (ref. 9–45)	102	40	70	61	92
AdoHcy, CSF (nmol/liter)	102 (ref. 1–29)	68	n.a.	n.a.	n.a.	n.a.
tHcy plasma (μmol/liter)	9.7 (ref. 5–15)	15	14.7 (ref. 5.5–17)	6	26	18
Adenosine, urine (mmol/mol creatinine)	7.1 (ref. < 1)	9.9 (ref. < 2)	n.a.	n.a.	n.a.	n.a.
Inosine, urine (mmol/mol creatinine)	2.7 (ref. < 3)	3.6	n.a.	n.a.	n.a.	n.a.
Xanthine, urine (mmol/mol creatinine)	6.8 (ref. < 30)	4.9 (ref. < 80)	n.a.	n.a.	n.a.	n.a.
Uric acid, plasma (μmol/liter)	469 (ref. 230–480)	321 (ref. 155–350)	n.a.	n.a.	n.a.	n.a.
Prolonged conjugated hyperbilirubinemia	+	n.a.	+	+	+	+
Alanine aminotransferase (U/liter)	241 (ref. 0–37)	447	200	245	106	400
INR	1.7 (ref. < 1.2)	1.6	n.a.	1.1	1.3	1.4
CK (U/liter)	705 (ref. 0–250)	276	190	normal	300–400	130
Liver biopsy	steatosis	n.a.	cholestasis	cholestasis	cholestasis	n.a.
Macrocephalus/frontal bossing	+/+	+/+	+/+	−/+	+/+	+/+
Epilepsy/age at debut	+/1 yr	+/1 yr	+/2 y 11 mo	+/10 mo	+/16 mo	+/2 yr
Age at diagnosis	23 yr	deceased at 10 y 9 mo	18 yr	11 yr	10 yr	7 yr

Maximum values are shown. Abbreviations are as follows: ref., reference value or range; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; CK, creatine kinase; CSF, cerebrospinal fluid; INR, international normalized ratio; Met, methionine; n.a., not analyzed; and tHcy, total plasma homocysteine.

and an increased normalized ratio (INR), in both cases. Liver biopsies performed at 10 months of age in the boy showed slight portal fibrosis and steatosis. Magnetic resonance imaging (MRI) of the brain showed cerebral atrophy with unspecific white-matter degeneration and secondary dilated ventricular systems in the boy. MRI of the brain was essentially normal at 14 months in his sister.

When the suspicion of ADK deficiency was raised, we used this information to identify four Malaysian individuals who were from two unrelated families and had a similar biochemical phenotype and clinical presentation (Table 1). These were two pairs of siblings, and the parents were first cousins in both cases. All had severe developmental delay and epilepsy, and all but one had macrocephalus with frontal bossing. Three individuals had cardiac defects; subject 4 had mild pulmonary stenosis and a small secundum atrial septal defect, subject 5 had a small secundum atrial septal defect, and subject 6 had a mild coarctation of the aorta. Two individuals had mild to moderate sensorineural hearing loss. GNMT deficiency had been considered, but sequence analysis of the corresponding gene did not reveal any pathogenic mutations.

The Regional Ethics Committee at Karolinska Institutet approved this study, and written informed consent was obtained from the parents.

Measurements of AdoMet and AdoHcy

AdoMet and AdoHcy in plasma and CSF were measured without derivatization essentially as described.[6] In brief, 500 μl of plasma or CSF samples were deproteinized with perchloric acid, and subsequently stable-isotope-labeled internal standards of both AdoMet and AdoHcy were added to the clear supernatant. Standard curves with increasing amounts of the analytes and fixed amounts of their internal standards were prepared for each set of

samples. Analytes were extracted from the perchloric supernatant via an anion-exchange solid-phase extraction procedure, and 10 μl of the final eluate was injected onto the LC-MS/MS. The LC-MS/MS, equipped with an electrospray interface, was operating in the positive multiple-reaction monitoring mode. The intra assay coefficients of variation for AdoMet and AdoHcy were <5%, and the inter assay coefficients of variation for AdoMet and AdoHcy were <8%.

Assay of SAHH Activity in Fibroblasts

Skin fibroblasts from the two Swedish siblings were cultured in Ham F10 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Fibroblasts were harvested by trypsination and washed three times with Hanks buffered saline solution (HBSS). The cell pellet was resuspended and lysed in 200 μl of a 50 mM potassium phosphate buffer containing 0.1% lubrol (pH = 7.4). The homogenate was centrifuged at 10,000 × *g* at 4°C for 10 min. The supernatant was kept on ice and used for the enzyme assay and protein determination. SAHH activity was measured by incubation of the homogenate with 25 μM AdoHcy for 30 min at 37°C and subsequent measurement of the amount of formed Hcy formed by LC-MS/MS (API3000, Applied Biosystems), essentially according to AB Sciex protocols.

Whole-Exome Sequencing

We used approximately 3–5 μg of genomic DNA from the two Swedish siblings to prepare paired-end libraries according to standard protocols supplied by Illumina (Illumina Inc., San Diego, CA). The libraries were subjected to exome enrichment via the in-solution SureSelect All Exon Kit (Agilent Technologies, Santa Clara, CA). The enriched DNA libraries were sequenced (2 × 76 bp) on a HiSeq 2000 sequencing system (Illumina). The sequencing data were mapped to the canonical chromosomes (chromosomes 1–22, X, Y, and M; UCSC genome browser) of the hg19 (GRCh37; downloaded from the UCSC genome browser) reference human genome via Mosaik (v. 1.0.1388); up to four mismatches were allowed. After removal of PCR duplicates (MosaikDupSnoop) and reads without a unique mapping location (MosaikSort), quality score recalibration and indel realignment were performed with the GATK package. Variants were extracted with the Maq model in SAMtools[7] and filtered by the following criteria: coverage ≥ 8×, consensus quality ≥ 20, SNP quality ≥ 20, and root-mean-square mapping quality ≥ 20. Identified variants were filtered against dbSNP build 131 (UCSC genome browser) and annotated on the basis of the UCSC knownCanonical transcript database (UCSC genome browser) via custom perl scripts. Any gene containing nonsynonymous (NS) or splice-site (SS) variants or coding indels (I) was considered a candidate gene under the dominant model. Under the recessive model, genes containing one or more homozygous or two or more heterozygous NS, SS, or I variants were considered candidate genes. An even more stringent homozygous model, demanding at least one homozygous NS, SS, or I variant, was also used. We used PolyPhen 2 to analyze the mutations located in the candidate genes and predict their effects on the protein.[8,9]

Molecular Modeling

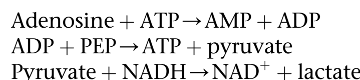
Images were obtained with the Molsoft MolBrowser 3.7-2a software.

Cloning, Expression, and Purification of Wild-Type and Mutant ADKs

Human *ADK* cDNA encoding the full-length protein (amino acids 1–345) and a C-terminal 6× His tag was optimized for expression in bacteria by gene synthesis. The corresponding DNA fragment was cloned between the NdeI and BamHI sites of the pET3a vector (Merck Biosciences). For the creation of mutant *ADK* versions, we used the QuickChange site-directed mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing. Protein expression in Rosetta 2(DE3)pLysS cells (Merck Biosciences) was induced by the addition of 0.8 mM isopropyl-1-thio-β-d-galactopyranoside to LB broth, and induction was maintained at 20°C for 20 hr. Protein was purified over a His-Select Ni^{2+} resin (Sigma–Aldrich, Stockholm, Sweden) according to the manufacturer's recommendations. After dialysis against 25 mM Tris-Cl (pH 8.0), 0.5 mM EDTA, 10% glycerol, 1 mM DTT, and 200 mM KCl, the purified recombinant ADKs were quantified via immunolotting. A calibration curve was made with commercial human ADK (NOVOCIB).

Determination of ADK Activity in Wild-Type and Mutant Recombinant Enzymes

Adenosine kinase activity of the recombinant proteins was assayed essentially as described[10] by enzymatic coupling of ADP formation resulting from the ADK reaction, to NADH oxidation:



$$\begin{aligned}&\text{Adenosine} + \text{ATP} \rightarrow \text{AMP} + \text{ADP}\\&\text{ADP} + \text{PEP} \rightarrow \text{ATP} + \text{pyruvate}\\&\text{Pyruvate} + \text{NADH} \rightarrow \text{NAD}^{+} + \text{lactate}\end{aligned}$$

The assay mixture (final volume 500 μl) contained 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 0.5 mM $MgCl_2$, 5 mM DTT, 100 μM NADH, 500 μM phosphoenolpyruvate, 1 mM ATP, 10 U/ml lactate dehydrogenase (Sigma), 6.8 U/ml pyruvate kinase (Sigma), and 4 ng/μl of purified recombinant ADK. Reactions were started by the addition of adenosine (ranging from 5–200 μM), and tracking the decrease of absorbance at 340 nm ($\varepsilon = 6.22 \times 10^3\ M^{-1} \times cm^{-1}$) on a Varian Cary 4000 Spectrophotometer (Agilent Technologies) at 37°C allowed measurement of NADH oxidation. The activity was expressed as μmoles of adenosine consumed/min × mg protein. All measurements were corrected for blanks containing no adenosine.

Results

SAHH Activity in Fibroblasts

An assay of S-adenosylhomocysteine hydrolase activity was established and used in fibroblasts from the two affected Swedish siblings. SAHH activity was 39.6 nmol/hr/mg protein for the brother and 30.8 nmol/hr/mg protein for the sister (reference range: 32.3–37.9 nmol/hr/mg), thus excluding SAHH deficiency.

Exome Sequencing Identifies *ADK* as a Candidate Gene in the Affected Swedish Siblings

Approximately 15 gigabases of sequence was generated per individual, resulting in an approximately 125-fold coverage of the 37 Mb targeted exome. More than 94%

Table 2. Identification of Candidate Genes by Exome Sequencing in the Two Siblings Affected by a Previously Undescribed IEM

	Sibling 1			Sibling 2			Siblings 1 and 2		
	Dom	Rec	Hom	Dom	Rec	Hom	Dom	Rec	Hom
NS/SS/I	4,539	2,789	2,103	4,452	2,782	2,111	3,750	2,270	1,639
Not in dbSNP131	424	62	26	422	49	21	213	28	13
Predicted to be damaging	204	17	10	195	17	8	100	10	5

Gene processing for the affected pair of siblings in the Swedish family. Filtering against databases of known variants (dbSNP 131) and application of the recessive model for genes present in both siblings reduced the number of genes more than 150-fold in all cases and almost 350-fold for the homozygous model. Additional information from PolyPhen 2 further reduced the gene count to a total of ten and five genes that overlapped between the two siblings under the recessive and the homozygous models, respectively. The gene containing the disease-causing mutations was present in the final list of five genes. Abbreviations are as follows: Dom, $\geq$ 1 heterozygous or homozygous variant; Rec, $\geq$ 1 homozygous or $\geq$ 2 heterozygous variants; Hom, $\geq$ 1 homozygous variant; NS, nonsynonymous variant; SS, splice-site variant; I, coding indel.

of targeted bases were sufficiently covered to pass our threshold for variant calling. Close to 130,000 variants were identified per individual; more than 90% were single-nucleotide variants, and fewer than 10% were indels. Each sibling was found to have at least one NS/SS/I variant in approximately 4,500 genes (dominant model) and two or more NS/SS/I variants in 2,800 genes (recessive model) (Table 2). Filtering against dbSNP build 131 to identify novel variants reduced the candidate gene pool 10-fold for the dominant model and 50-fold for the recessive model. When we considered only variants shared in both siblings, the pool of candidate genes under a dominant model was reduced to 213, and under our recessive model, the number of candidate genes was reduced to 28. Using PolyPhen 2 to predict the effect of a mutation generated a list of ten genes with mutations predicted not to be benign under the recessive model, a total reduction of almost 450-fold. These genes were *ADK* (RefSeq number NM_001123.3), *FAM75C1* (NM_001145124), *FCGBP* (NM_003890), *FLG* (NM_002016), *FRG2B* (NM_001080998), *FRG2C* (NM_001124759), *GGT1* (NM_013430), *HRNR* (NM_001009931), *KRTAP9-9* (NM_030975), and *ZNF141* (NM_003441). We had no information of consanguinity in the family. However, both parents originate from the same rural area in Sweden, where genetic diversity is reduced. This increases the probability that both parents are heterozygous carriers for the same disease-causing variant. There were five genes containing novel homozygous variants predicted to be damaging. These were *ADK* (NM_001123.3), *FAM75C1* (NM_001145124), *FRG2B* (NM_001080998), *GGT1* (NM_013430), and *HRNR* (NM_001009931). Among these, *ADK*, encoding the enzyme adenosine kinase, stood out because blockage of this enzyme would be predicted to disrupt the methionine cycle.

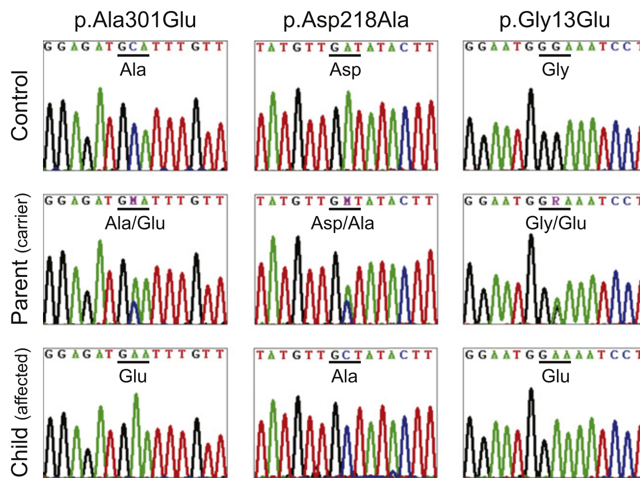


Figure 2. Sanger-Sequencing Traces of the Three *ADK* Missense Mutations Causing Adenosine Kinase Deficiency
The first column shows results verifying the mutation detected by exome sequencing in two affected Swedish siblings: a homozygous C>A transversion at nucleotide 902 resulted in a p.Ala301Glu amino acid substitution in adenosine kinase. The second and third columns show the two homozygous mutations detected in two Malaysian families with ADK deficiency: an A>C transversion at nucleotide 653 caused a p.Asp218Ala amino acid substitution, and a G>A transition at nucleotide 38 led to a p.Gly13Glu amino acid substitution. In all cases, the control, wild-type sequences are shown on top, a heterozygous parent is shown in the middle row, and the homozygous mutations detected in the affected children are displayed in the bottom row.

Validation of Adenosine Kinase Deficiency in the Affected Individuals

To validate our finding from exome sequencing, we used DNA isolated from family members and analyzed exon 10 of *ADK* (National Center for Biotechnology Information GeneID 132) by Sanger sequencing (Figure 2). The previously identified c.902C>A transversion causing a p.Ala301Glu missense mutation was confirmed in homozygous form in both affected siblings. Both parents were heterozygous for the mutation, which was not present in 105 unaffected Swedish controls. To verify impaired adenosine kinase function in vivo, we measured urinary adenosine levels. Frozen samples from 2001 were retrospectively analyzed, and adenosine was elevated to 3.5 mmol/mol creatinine in the boy (reference value < 1) and 9.9 mmol/mol creatinine in his sister (reference value < 2). Analysis of a fresh sample from the affected boy at 23 years of age showed an adenosine concentration of 7.1 mmol/mol creatinine (reference value < 1). All other purines and pyrimidines were essentially normal. DNA from the two Malaysian families was subsequently obtained, and all coding exons of *ADK* were analyzed by Sanger sequencing. A c.653A>C transversion causing a p.Asp218Ala mutation was found in homozygous form in both siblings from the first family, and a c.38G>A transition causing a p.Gly13Glu mutation was found in homozygous form in both affected siblings from the

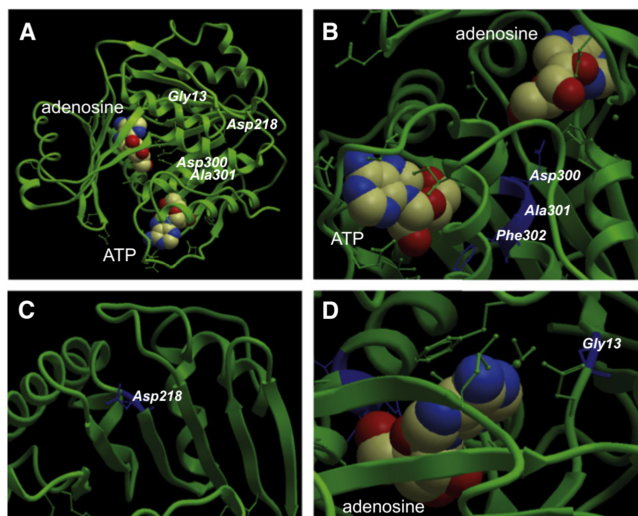


Figure 3. Locations of the Mutated Residues in Human ADK
(A) Overall view of human ADK, showing the relative position of the three mutated residues in the molecule and the catalytically important Asp300. Two adenosine molecules, one occupying the adenosine-binding site and the other the ATP-binding site, are depicted.
(B) Close-up image showing the position of Ala301. The mutated amino acid sits between two crucial residues. Asp300 is ADK's active site responsible for the deprotonation the 5′-OH group of adenosine, which in turn attacks the gamma-phosphate of ATP. Phe302 contributes to the binding of ATP. These three residues are located in an alpha-helix between the two binding sites. Replacing Ala301, a residue with a short, neutral side chain, with glutamic acid, containing a longer, charged side chain, is likely to have deleterious consequences for enzyme function.
(C) Close-up image showing the location of residue Asp218 in the central beta-sheet domain of the molecule. Asp218 sits at the N terminus of beta-strand 11, which is part of a beta-sheet domain that forms the core of the overall ADK structure.
(D) Close-up image showing the proximity of residue Gly13 to the binding site for adenosine. The substitution of glycine, which has only a hydrogen atom for a side chain, for glutamic acid, which has a long, charged side chain, is likely to compromise the binding of adenosine to ADK.
The numbering is based on the short isoform of ADK (isoform a; transcript NM_001123.3; protein NP_001114.2), the crystal structure of which has been resolved.[12]

second family (Figure 2). As expected, all parents carried one mutation in heterozygous form. The locations of the mutated residues were visualized in a model of human ADK (Figure 3). All mutations were located in structurally and/or functionally critical positions.

Assay of Adenosine Kinase Activity in Wild-Type and Mutant Recombinant Enzyme

In order to test the functional effect of the mutations found in our affected subjects, we expressed recombinant forms of wild-type ADK, as well as ADK containing each of the mutations, in *E. coli*, purified them, and tested them for their capacity to phosphorylate adenosine over a wide range of adenosine concentrations. The K_m of human ADK for adenosine at pH 7.5 has been estimated to 0.15 μM, and maximum activity has been estimated at

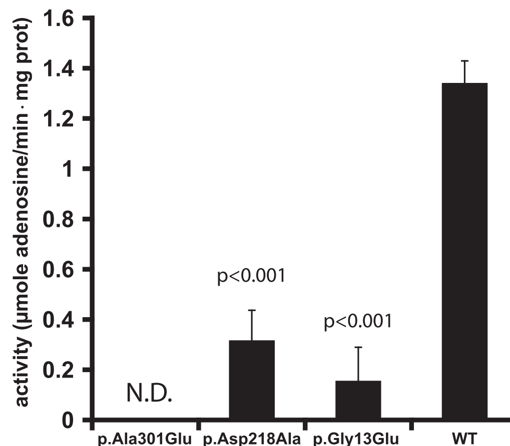


Figure 4. Adenosine Kinase Activity from Recombinant Wild-Type and Mutant Enzymes
Recombinant forms of wild-type ADK, as well as ADK containing each of the mutations detected in individuals with ADK deficiency, were expressed in *E. coli*, purified, and tested for their capacity to phosphorylate adenosine. The graph shows the mean value + 1 standard deviation of three independent measurements carried out with 5 μM adenosine as described in the Subjects and Methods. All mutants displayed significantly impaired activity in comparison to the wild-type enzyme (two-tailed Student's t test). N.D. denotes not detectable; WT denotes wild-type.

0.5 μM. At higher concentrations, adenosine might also occupy the ATP-binding site in ADK, which in turn leads to an inhibition of enzyme activity.[10] The activity of our recombinant wild-type ADK closely resembled the pattern previously described for the native form. The spectrophotometric method used does not allow one to accurately assay ADK activity for adenosine concentrations below 5 μM, and therefore K_m and V_{max} values could not be obtained. Nevertheless, the measurements obtained still allowed us to effectively compare the activity of the different mutants with that of the wild-type form. The p.Ala301Glu mutant showed essentially no activity (Figure 4). The p.Asp218Ala mutant showed some residual activity, amounting to about 20% of the wild-type at 5 μM adenosine. The p.Gly13Glu mutant showed traces of activity (about 10% at 5 μM adenosine compared to the wild-type).

Discussion

Methionine is an essential amino acid that is not metabolized via transamination as are most other amino acids. The methionine cycle comprises a series of enzymatic conversions, including adenosylation of Met by methionine adenosyl transferases to AdoMet, donation of the methyl group by one of many methyltransferases to a wide range of methyl acceptors generating AdoHcy, and subsequent hydrolysis by SAHH to generate adenosine and the demethylated analog of Met, Hcy (Figure 1). Hcy

is subsequently either remethylated back to Met or irreversibly transsulfurated via cystathionine to cysteine.

The siblings in our study had increased levels of Met, AdoMet, and AdoHcy, indicating disruption of the methionine cycle between AdoHcy and Hcy, but SAHH deficiency was ruled out. Exome sequencing produced 10 (5 homozygous) candidate genes that conformed with a recessive model and were shared in both siblings. Among these, *ADK* stood out because it could be anticipated to influence the function of SAHH (Figure 1). Adenosine kinase (ADK; EC 2.7.1.20) is an abundant nucleoside kinase that catalyzes the phosphorylation of the purine nucleoside adenosine at the 5′ position in an ATP-dependent manner. Two isoforms of ADK have been identified in humans. Both are identical except for a 17-residue extension at the N terminus of the long isoform. The short isoform is cytosolic, whereas the long isoform recently has been localized to the nucleus.[11] The crystal structure of the human ADK (short isoform) has been resolved.[12] ADK catalyzes the phosphorylation of adenosine to produce AMP.[13,14] Adenosine is produced in the methionine cycle together with Hcy via hydrolysis of AdoHcy by SAHH. However, the equilibrium of this reaction lies far in the direction of condensation, producing rather than degrading AdoHcy. The thermodynamics thus favor production of AdoHcy, and AdoHcy will only be hydrolyzed if the reaction products, adenosine and Hcy, are transported or removed enzymatically.[15] Thus, an adenosine increase due to impaired removal by ADK would be predicted to result in increased AdoHcy levels.

An *Adk* knockout mouse showing no prenatal phenotype but developing progressive microvesicular liver steatosis from approximately postnatal day two has been generated.[16] The mice died within the first 8 days, and on the basis of these results, ADK deficiency was proposed as a putative causative factor for fatal neonatal hepatic steatosis. However, the mice also displayed an abnormal postnatal development from day 3, despite normal feeding and nursing by the mothers; there was a retardation in body weight development, a delay in the time to eye opening, and intermittent periods of apnea as well as an abnormal temperature regulation. Both AdoMet and AdoHcy were significantly elevated and the concentrations of total adenine nucleotides were decreased in liver homogenates. Taken together, the human and mouse phenotypes are strikingly similar, although our affected individuals presented with a predominantly neurological phenotype in combination with moderate liver dysfunction, whereas the mice died too early to allow a full neurological evaluation. During embryonic development, adenosine is mainly degraded into inosine by adenosine deaminase (ADA), but this reaction is not pronounced in postnatal liver, where ADA is expressed at very low levels.[17] Postnatally, the primary route of adenosine metabolism is believed to be conversion to AMP by ADK.[12,18] This probably explains the normal prenatal development and the postnatal onset of symptoms in both mice and humans. Mutations in *ADA* cause severe combined immune deficiency (SCID [MIM 102700]), and increased levels of both adenosine and 2′-deoxyadenosine affect predominantly, but not exclusively, the immune system.[19]

Several putative pathogenetic mechanisms might contribute to the phenotype of ADK deficiency. Adenosine is toxic to mammalian cells; for example, it is associated with inhibition of the immune response, coronary vasodilation, delayed neurotransmission, and abnormal hormone secretion. Adenosine is also considered a cell-protective molecule and is increased during inflammation and hypoxia, for example. Physiologically, the dephosphorylation of AMP by 5′-nucleotidase to adenosine and phosphate and the recycling of adenosine back to AMP by ADK forms an ATP-consuming cycle that is considered an important regulator of adenosine levels (the adenosine futile cycle). Adenosine is a component of many vital enzyme cofactors, such as FADH, NAD(P)H, and coenzyme A, and its biochemical effects also include activation of adenylate cyclase, inhibition of pyrimidine biosynthesis, and diminution of phosphoribosyl pyrophosphate synthesis. In addition to toxic effects of increased adenosine, decreased levels of adenine nucleotides are likely to be detrimental for cellular metabolism and mitochondrial function. As one example, AMP-activated protein kinase (AMPK) has been termed a "super metabolic regulator" because of its effect on multiple pathways, including those involved in glucose and fatty acid metabolism.[20]

Adenosine accumulation leads to reversal of the SAHH reaction, and this reversal causes increased AdoHcy levels and disrupts the methionine cycle (Figure 1).[21] Methylation involving AdoMet as the methyl group donor takes place in all cells and this housekeeping metabolism comprises the methylation of a large variety of substrates, including DNA, mRNA, tRNA, proteins, lipids, catechols, indoles, guanidinoacetic acid, and many other compounds. Mammalian methyltransferases are considered to be subject to product inhibition by AdoHcy.[22,23] Thus, ADK deficiency is predicted to interfere with a wide range of methyltransferase reactions.

Up to now, four IEMs have been known to present with hypermethioninemia as a result of enzyme deficiencies that directly interfere with Met or Hcy metabolism.[5] The enzymes deficient in these disorders are MAT I/III, GNMT, SAHH, and CBS. Here we have described ADK deficiency, a previously undescribed IEM that disrupts the methionine cycle through a principally different mechanism. The finding sheds light on a functional link between the methionine cycle and adenosine metabolism, and the disorder presents with a severe, slowly progressive encephalopathy with epilepsy, macrocephalus and abnormal liver function. Hypermethioninemia is occasionally identified incidentally, as well as in neonatal screening programs aimed at diagnosing CBS deficiency.

Because ADK deficiency leads to grossly abnormal development in affected children, awareness of this disorder and discrimination against more benign conditions is important.

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Web Resources

The URLs for data published herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
UCSC genome browser, http://genome.ucsc.edu/
Mosaik, http://code.google.com/p/mosaik-aligner/
GATK package, http://www.broadinstitute.org/gsa/wiki/index.php/The_Genome_Analysis_Toolkit
National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/
Molsoft, www.molsoft.com
International Union of Biochemistry and Molecular Biology (IUBMB), http://www.chem.qmul.ac.uk/iubmb/

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