Autosomal Dominant Glut-1 Deficiency Syndrome and Familial Epilepsy

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Glut-1 deficiency syndrome was first described in 1991 as a sporadic clinical condition, later shown to be the result of haploinsufficiency. We now report a family with Glut-1 deficiency syndrome affecting 5 members over 3 generations. The syndrome behaves as an autosomal dominant condition. Affected family members manifested mild to severe seizures, developmental delay, ataxia, hypoglycorrhachia, and decreased erythrocyte 3-*O*-methyl-D-glucose uptake. Seizure frequency and severity were aggravated by fasting, and responded to a carbohydrate load. Glut-1 immunoreactivity in erythrocyte membranes was normal. A heterozygous R126H missense mutation was identified in the 3 patients available for testing, 2 brothers (Generation 3) and their mother (Generation 2). The sister and her father were clinically and genotypically normal. In vitro mutagenesis studies in *Xenopus laevis* oocytes demonstrated significant decreases in the transport of 3-*O*-methyl-D-glucose and dehydroascorbic acid. *Xenopus* oocyte membranes expressed high amounts of the R126H mutant Glut-1. Kinetic analysis indicated that replacement of arginine-126 by histidine in the mutant Glut-1 resulted in a lower V_{max}. These studies demonstrate the pathogenicity of the R126H missense mutation and transmission of Glut-1 deficiency syndrome as an autosomal dominant trait.

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D-glucose is an essential fuel for metabolism in mammalian cells and the predominant fuel source for the brain. Transport of glucose across tissue barriers is mediated by stereospecific transporter proteins. Two families of glucose transporters have been identified in humans: the Na^+-glucose cotransporters, which actively transport glucose, and the facilitative glucose transporters (Gluts), which enable diffusion of glucose across tissue barriers. Seven functional transporter proteins (Glut-1 through Glut-5, Glut-8, Glut-9) with distinct tissue distributions, subcellular localizations, and transport kinetics[1–4] have been identified in the GLUT family. The *GLUT-1 (SLC2A1, MIM 138140)* gene encodes a protein (Glut-1) of 492 amino acids. The gene maps to the short arm of chromosome 1 (1P31.3→35),[5] is 35kb in length, and contains 10 exons.[6] Glut-1, originally called the HepG2/erythrocyte/brain transporter,[7] was the first identified member of this family. It is constitutively expressed in most tissues and selectively expressed in erythrocytes, brain microvessels, and astroglia. Glut-1 has two distinct molecular forms with apparent molecular weights of 55 and 45 kDa. These two forms are encoded by the same gene and differ only in their extent of glycosylation.[8] The 55 kDa form is found predominantly in the endothelial cells of brain microvessels and erythrocytes, where it is the principal (if not exclusive) glucose transporter. The 45 kDa form is detected in most cells (including glia, neurons, and choroid plexus);[9] it may be responsible for basal constitutive glucose uptake into these tissues. The asymmetric distribution of Glut-1 on the luminal and abluminal surfaces of brain microvessels has been shown by immunogold electron microscopy in humans, rabbits, rats, rhesus monkeys, squirrels, and vervet monkeys.[10,11]

Other solutes that can diffuse through Glut-1 are H_2O,[12] galactose,[13,14] dehydroascorbic acid (DHA),[15–17] and perhaps glycopeptides.[18] Humans cannot synthesize vitamin C.[19] After transport across the blood–brain barrier, DHA is irreversibly reduced and trapped in the brain as ascorbic acid.[16]

In 1991, De Vivo and colleagues[20,21] first described 2 children with Glut-1 deficiency syndrome (Glut-1 DS). Since then, we have diagnosed about 50 patients

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clinically and reported 20 patients with various mutations.[22–24] Glut-1 DS is characterized by infantile seizures, developmental delay, acquired microcephaly, ataxia, hypoglycorrhachia, reduced cerebrospinal fluid (CSF)/blood glucose ratio (~0.33, normal ratio ~0.65),[21] and decreased glucose uptake into erythrocytes (46 ± 8% of control).[25] Seizures respond to a ketogenic diet, but patients continue to experience peculiar paroxysmal episodes (such as limpness, ataxia, hemiparesis, and confusion) and neurobehavioral disturbances.[21] Glut-1 immunoreactivity in the erythrocyte membrane may be normal or 50% reduced.[22,23] Mutational analysis of the *GLUT-1* gene has revealed either hemizygosity or heterozygosity for missense, nonsense, insertion, deletion, and splice site mutations. These genotypic features suggest that Glut-1 DS may be inherited as an autosomal dominant trait.[22–24] We now describe a novel missense mutation in the *GLUT-1* gene associated with the first reported familial multigenerational case of Glut-1 DS.[26] A second family with autosomal dominant transmission has also been reported.[27]

Patients and Methods

Investigatory studies on blood were exempted from full review and approved by the Columbia University Institutional Review Board. Informed consent was obtained from the parents and patients.

Case Reports

PATIENT 1. After an uneventful term pregnancy, the newborn male had normal body weight and head circumference. The head grew normally along the 75th percentile until age 12 months. Deceleration of head growth followed. Neurological development was delayed. He sat at age 15 months, walked at 3 years, and spoke after 3 years. He attended a special school for the neurologically disabled. At age 3 months, he had his first seizure, described as myoclonic jerking of the shoulders and arms, nodding of the head, rolling of the eyes, limpness, and impaired consciousness. Cognitive and motor skills declined, and daily seizures were correlated with increasing intervals between meals, most notably in the morning before breakfast. Hypoglycorrhachia was noted at age 9 months but not pursued further. His neurological functioning improved after a meal, and seizures were decreased after eating sweets. Phenobarbitone and valproic acid were ineffective. At age 16 years, weight was at the third percentile, height below the third percentile, and head circumference at the 25th percentile. He was friendly and content. His vocabulary was limited to 20 words, speech was dysarthric and slow, muscle tone was decreased, and gait was ataxic. He walked only a few steps unsupported. Tendon reflexes were present, and Babinski signs were absent.

PATIENT 2. The younger brother of Patient 1 was born at term after an uneventful pregnancy. Weight, length, and head circumference were normal. His head circumference gradually decelerated from the 75th percentile at birth to the 50th at age 8 years. His neurological functioning was not as delayed as that of his elder brother; he sat unsupported at age 6 months and walked at age 14 months. He spoke his first words in his second year. He attended a special school for the disabled. At age 6 months, he had his first seizure. Daily seizures followed with impaired consciousness, rolling of the eyes, head nodding, jerking of the shoulders and arms, and sudden loss of tone. Before breakfast, he had a dazed expression, slow motor and mental responses, slurred speech, ataxia, and dyskinetic movements. His neurological performance improved postprandially.

At age 8 years, he was content and friendly, hypotonic, clumsy, and slightly dyskinetic. He was just able to hop on one foot. Tendon reflexes were normal, and Babinski signs were absent. The parents noted increased seizures and worsening neurological performance during short fasting intervals and requested frequent feedings every 2 hours at school.

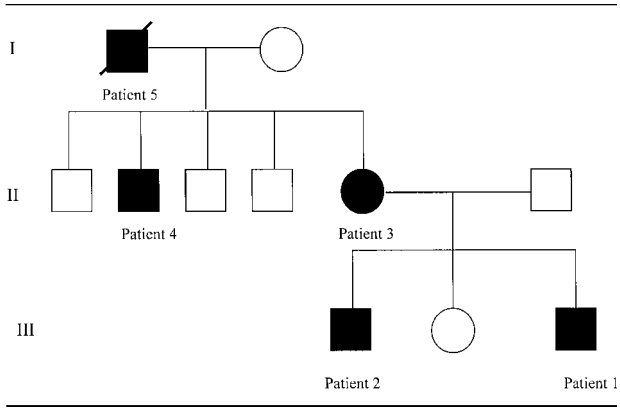


Fig 1. Pedigree. The deceased grandfather (Patient 5) *and his affected son* (Patient 4) *were clinically affected, as determined by history, and were not available for the mutation screening.* Patients 1 *to* 3 *were clinically affected and confirmed genotypically. The ages of Patients 1 to 5 are 16, 8, 48, 51, and 78 years, respectively.*

PATIENT 3. The mother of Patients 1 and 2, age 48 years, had suffered seizures from childhood with unresponsiveness, impaired consciousness, head nodding, and sudden loss of tone. As with her sons, seizures occurred predominantly in the morning before breakfast. Her neurological performance and seizures improved after a meal, especially after eating sweets. She learned to eat every 2 to 3 hours during the day, and to awaken at night to eat some sweets. Her husband noted her speech to be slow, slurred, and dysarthric before meals. Coffee further impaired her performance. She obtained a high school diploma, and has driven a car for 20 years without an accident.

Her head circumference was normal, body weight 73kg, and height 168cm. She was clumsy; otherwise, her neurological examination was normal, if performed within a few hours of eating. She was an exceptionally friendly person. Her cognitive skills were slightly below average.

Family History

The pedigree is shown in Figure 1. There is a healthy sister, age 12 years. The mother (Patient 3) has 4 older brothers: 3

are healthy, while the fourth (Patient 4) had similar seizures in childhood and altered mentation after fasting. Purportedly, he is more severely affected than his sister, and he refused frequent feeding as a child. He is now employed as a forest worker in rural Germany, and declined clinical examination or laboratory investigations.

The grandfather (Patient 5) of Patients 1 and 2 was in charge of a telegraph office in northern Germany. No neurological signs or symptoms were reported, but medical records were limited. However, it is alleged that his wife used to serve him honey at bedside in the morning to improve his alertness and neurological functioning. At the age of 78 years, he died from a stroke.

*Erythrocyte 3-*O*-Methyl-*D*-Glucose Uptake Studies*

Investigations of uptake of 3-*O*-methyl-D-glucose (3-OMG) into red blood cells (RBCs) were performed as described elsewhere.[25]

Western Blot Analyses of Red Blood Cell Membrane Ghosts

Erythrocyte membranes were isolated from the father and Patients 1, 2, and 3. Western blots were performed as previously described;[22] Glut-1- and band 3-specific signals were visualized by chemiluminescence reactions. Glut-1 and band 3 signals were quantified and transformed into digital values using a densitometer equipped with *ImageQuant* software (Molecular Dynamics, Sunnyvale, CA). The densities of band 3 signals served as an internal control for protein loading. The corresponding band 3 densities were used to normalize Glut-1 densities.

Mutational Analysis of the GLUT-1 *Gene*

White blood cells were used to extract genomic DNA from patients and their parents. Mutational analysis of *GLUT-1* was performed as described previously.[24] The mutation identified was confirmed by sequencing of an independent polymerase chain reaction (PCR) product.

Mutagenesis and cRNA Preparation from cDNA Templates

A 1,913 bp Eco 47 III and Hind III *GLUT-1* cDNA fragment derived from pcDNA3 (Invitrogen, Carlsbad, CA) was subcloned into a customer plasmid (pM) containing fragments of 5′- and 3′-untranslated regions of *Xenopus* β-globin cDNA, and will be described elsewhere. Plasmid insert was confirmed by direct DNA sequencing with appropriate primers. Mutation of R126H was introduced by PCR. Forward and reverse mutagenic primers were AGGGCGCAATTGCGGTACCCAGCTTGCTGG (forward 1), TTTGAGATGCTGATACTGGGCCACTTCATCATCGG (forward 2), CCGATGATGAAGCGGCCCAGGATCAGCATCTCAAA (reverse 1), and GCCTGCAACGGCAATGGCAGCTGGACGTGG (reverse 2). PCR was conducted on the pM-*GLUT-1* plasmid DNA using the above primers for 35 cycles with denaturation at 95°C for 30 seconds, annealing at 58°C for 1 minute, and elongation at 72°C for 2 minutes. The PCR fragment of the right size was purified from the gel and cut with StuI and MunI. The gel-purified StuI and MunI fragments were then directionally ligated into the StuI and MunI sites of pM-*GLUT-1*. The presence of mutation R126H was confirmed by DNA sequence analysis. Capped, runoff cRNA transcripts of wild type and mutant *GLUT-1* were synthesized from pM-*GLUT-1* constructs after linearization at a unique NotI site using the mMESSAGE mMACHINE kit (Ambion, Austin, TX). cRNAs were quantified by incorporation of ^{3}H-UTP tracer in the transcription reaction, and their integrity was verified by denaturing agarose electrophoresis. cRNAs were stored at −80°C in 100mM KCl until the time of dilution for injection.

Xenopus laevis *Oocyte Studies*

Oocytes were prepared as previously described.[28] The largest undamaged oocytes (stages V and VI) were transferred to Barth's solution, incubated overnight at 18°C, and injected the following day. Oocytes were injected with 50nl water and 50nl wild-type or mutant cRNA (1μg/μl) and kept in Barth's medium at 18°C for 72 hours.

X. laevis oocyte transport assays were performed as described by Due and colleagues,[29] with modifications. In the zero-trans and equilibrium exchange influx studies, parallel assays were undertaken using oocytes injected with water as a control. These control transport rates were subtracted from transport rates by oocyte-expressing R126H mutant and wild-type Glut-1. Only net transport rates are shown.

Zero-trans influx of 3-OMG was initiated by incubating five oocytes in 1ml of Barth's solution containing 2.5μCi of ^{14}C 3-OMG and unlabeled 3-OMG at concentrations of 1 to 50mM, at a constant 179.1mOsm/L achieved by adding 1M sucrose solution. After 2 minutes, the transport assay was stopped by rapidly washing the oocytes 4 times in 2ml ice-cold Barth's solution, containing 0.2mM phloretin and 50mM 3-OMG. After the final wash, each oocyte was transferred into a scintillation vial containing 0.5ml 0.1% sodium dodecyl sulfate (SDS) and lysed by vortexing vigorously. Five milliliters of Hionic-Flour scintillation fluid (Packard Bioscience, Groningen, The Netherlands) were added to each vial, mixed by vortex, and counted with a Packard TR2300 Scintillation Counter.

Equilibrium exchange influx of 3-OMG into *X. laevis* oocytes was performed after overnight incubation at 18°C in 1ml of Barth's solution containing unlabeled 3-OMG at concentrations of 1 to 50mM. Osmolarity was maintained at 179.1mOsm/L by adding 1M sucrose. Transport assays were done as described above.

Zero-trans efflux of 3-OMG into *X. laevis* oocytes was also performed after overnight incubation at 18°C in 1ml of Barth's solution containing ^{14}C 3-OMG and unlabeled 3-OMG at concentrations of 1 to 50mM. Osmolarity was maintained at 179.1mOsm/L by adding 1M sucrose. Immediately before the efflux assay, oocytes were quickly washed in 20ml Barth's solution and transferred to a scintillation vial containing 0.5ml of Barth's solution. After 2 minutes, oocytes were transferred to another scintillation vial. 0.5ml of 0.1% SDS was added to both vials and mixed by vortex. Hionic-Flour scintillation fluid (5ml) was added before counting.

Zero-trans influx of DHA into *X. laevis* oocytes was performed by adding ascorbate oxidase (0.1–10 units) to L-[1-^{14}C]-ascorbic acid (DuPont NEN, Boston, MA) and incu-

bating for 15 minutes at room temperature to generate L-[1-^{14}C]-DHA, as previously described.[30] Solutions were prepared immediately before the assay. Influx was initiated by incubating 5 oocytes in 1ml of Barth's solution containing L-[1-^{14}C]-DHA at concentrations of 50 to 800μM. After 2 minutes, the assay was stopped; washing and counting were performed as described for the zero-trans influx of 3-OMG.

Ethylidene glucose inhibition of 2-deoxy-D-glucose (2-DOG) uptake was performed by incubating 5 to 10 oocytes in 1ml of Barth's solution containing 2μM ^{3}H 2-DOG with ethylidene glucose at concentrations of 0 to 50mM. After 10 minutes, the assay was stopped and oocytes were processed as described for the zero-trans influx of 3-OMG.

Confocal immunofluorescence microscopy permitted detection of Glut-1 in frozen *X. laevis* oocyte sections as described by Garcia and colleagues,[31] using as primary antibody goat polyclonal IgG (Glut-1 C20; Santa Cruz Biotechnology, Santa Cruz, CA), and as secondary antibody Alexa Fluor 568 conjugated donkey antigoat IgG (Molecular Probes, Eugene, OR). Confocal microscopy was performed at the Optical Microscopy Facility at Columbia University.

Membranes from injected oocytes were prepared by a method modified from Garcia and colleagues.[31] Briefly, oocytes were suspended in 50μl/oocyte homogenization buffer [10mM HEPES (pH 7.9), 83mM NaCl, 1.0mM $MgCl_2$, 0.5mM phenylmethylsulfonyl fluoride, 5μg/ml pepstatin A, 5μg/ml soybean trypsin inhibitor, 5μg/ml leupeptin] and homogenized with 20 strokes in a glass-Teflon homogenizer. After disruption of oocytes with a pipette, oocyte ghosts were washed free of yolk as previously described.[31] Washed oocyte ghosts were homogenized and further processed to obtain purified plasma membrane.

Western blot analysis was performed by subjecting 10μg of the prepared total membrane and purified plasma membrane samples from wild type R126H-, and H_2O-injected oocytes to 4% to 20% SDS-polyacrylamide gradient gel electrophoresis, as described in Western blot analyses of RBC membrane ghosts. Glut-1 signals were quantified and transformed into digital values using a densitometer equipped with *ImageQuant* software (Molecular Dynamics). The densities of wild type Glut-1 signals in purified oocyte membrane were used to normalize R126H mutant Glut-1 densities.

Results

Laboratory Studies

Cranial magnetic resonance imaging revealed slightly enlarged ventricles in Patient 1, and no abnormalities in Patient 2.

Electroencephalography usually showed normal results in both brothers during their first 3 years of life. Occasional tracings showed generalized spike-wave discharges. After age 3 years, background slowing and multifocal and generalized, irregular, high-amplitude spikes and spike-wave discharges were observed, usually correlating with myoclonic jerks and head nodding. More recent tracings showed improvement with resolution of epileptiform discharges 1 to 2 hours after eating.

Patients 1 and 2 were examined using the German manual of the Wechsler Intelligence Scale for Children-Revised (WISC-R)[32] at the ages of 16 and 8 years, respectively. WISC-R Verbal Scale IQ and Performance Scale IQ in Patient 1 were below 44, consistent with severe mental disability. Patient 2 showed a WISC-R Verbal Scale IQ of 66, and a Performance Scale IQ of 57, consistent with mild cognitive impairment.

Blood glucose, lactate, and ammonia values were repeatedly normal in Patients 1 and 2, as were urine organic acids and amino acids. CSF study results are summarized in Table 1. Patients 1 to 3 had decreased CSF glucose values. Blood glucose values, measured simultaneously, were normal, and the resulting CSF/blood glucose ratio was lowered to values below 0.44 (normal ~0.65). Hypoglycorrhachia had been found in Patient 1 at age 9 months, but was not pursued further. Clinical and laboratory features are summarized in Table 1.

3-OMG Uptake into Erythrocytes

Glucose zero-trans influx values were 72% in Patient 1, 56% in Patient 2, and 71% in Patient 3 compared with the father. Kinetic analysis revealed V_{max} values of 1,111 (56%), 1,250 (63%), 1,250 (63%), and 2,000 (100%) fmol/sec in 10^6 RBCs for Patients 1 to 3 and the father. K_m values for Patients 1 to 3 and the father were similar at 3.7, 3.3, 2.8, and 3.6mM.

Western Blot Analyses of Red Blood Cell Ghosts

Western blots, performed on erythrocyte membranes from Patients 1 to 3 and the father were normal. Densitometric quantification of signals from immunoreactive Glut-1, normalized to band 3 signals, is expressed as a percentage of the paternal expression level: Patient 1, 74.5 ± 5% (n = 3); Patient 2, 88.4 ± 10.3% (n = 3); Patient 3, 108.9 ± 12.1% (n = 3); and the father, 100% as the reference value.

Mutational Analysis of GLUT-1

Screening the promoter region, the 10 coding exons and the corresponding intron–exon boundaries of the *GLUT-1* gene uncovered a heterozygous missense mutation, R126H, in exon 4 at nucleotide 556 due to a G-to-A transition in Patients 1 to 3. The father and daughter were also normal at this locus. Silent substitutions were also found in this family. The results of mutational analysis are summarized in Table 2.

Mutagenesis Studies

The pathogenicity of the R126H missense mutation was confirmed by introducing this mutation into the *GLUT-1* cDNA and inserting the mutant cRNA into

Table 1. Summary of Clinical and Laboratory Features

	Patient 1	Patient 2	Patient 3
Gestation	42 weeks	40 weeks	Not known
Birth weight	P75	P90	Not known
Birth FOC	P75	P75	Not known
Somatic growth	P3 (weight), <P3 (height), P25 (FOC)	P25 (weight), P25 (height), P50 (FOC)	P90 (weight), P75 (height), P75 (FOC)
Mental development	Severe retardation	Moderate retardation	Slightly subnormal
Motor development	Moderate retardation	Slightly subnormal	Normal
Movement disorder	Severe, ataxia-dystonia	Mild, ataxia-dystonia	Clumsiness
Seizures	Nodding, eye rolling, jerking, atonia, impaired consciousness	Same as Patient 1	Nodding, atonia, impaired consciousness
Neurological worsening after fasting	+++	+++	+
EEG	Slowing, focal spikes, spike-wave discharges	Same as Patient 1	Not done
MRI of head	Enlarged ventricles	Normal	Not done
Age of patient at lumbar puncture	(1) 9 mo (2) 16 yr	8 yr	48 yr
CSF glucose[a]	(1) 30mg/dl (2) 30mg/dl	33mg/dl	38mg/dl
Blood glucose[b]	(1) 88mg/dl (2) 68mg/dl	95mg/dl	98mg/dl
Ratio CSF/blood glucose[c]	(1) 0.34 (2) 0.44	0.34	0.38
CSF lactate[d]	(1) Not done (2) 0.9mmol/L	0.9mmol/L	1.1 mmol/L

[a]Normal (mean ± SD) 62.5 ± 0.96 mg/dl.
[b]Normal 60–100 (child), 70–105 (adult).
[c]Normal 0.65 ± 0.01.
[d]Normal 1.3 ± 0.07.

FOC = fronto-occipital circumference; P25 = 25th percentile of age; + to +++, mild to severe; EEG = electroencephalography; MRI = magnetic resonance imaging; CSF = cerebrospinal fluid.

an *X. laevis* oocyte expression vector. The results of 3-OMG and DHA transport assays in wild type Glut-1 and R126H mutant studies are shown in Figure 2. The zero-trans influx, efflux, and equilibrium exchange for 3-OMG and the zero-trans influx for DHA were significantly decreased in the R126H mutant experiments. Kinetic data were normalized based on the relative amount of Glut-1 in the purified plasma membrane (see Fig 2). Confocal immunofluorescence microscopy of *X. laevis* oocytes injected with mutant R126H and stained for Glut-1 by indirect immunofluorescence showed similar patterns of labeling for Glut-1 in the cytoplasm and plasma membrane. The ratio of mutant R126H Glut-1 immunoreactivity in the purified plasma membrane to the total membrane was 10.2%, similar to the wild type Glut-1 immunoreactivity (11.3%; Fig. 3). The exofacial ligand, ethylidene glucose, inhibited 3-OMG transport activity of R126H mutant Glut-1 and wild type Glut-1 (Fig 4). The apparent affinities of ethylidene glucose for the exofacial transport site of wild type and R126H mutant Glut-1 were determined by analysis of inhibitor

Table 2. Mutational Analysis of GLUT-1 *Gene*

Subject	−173delA (E1)	GCT → GCC@224 A15A (E2)	CGC → CAC@556 R126H (E4)	TGC → TGT@578 C133C (E4)	CCG → CCA@767 P196P (E5)
Patient 1	−/−	−/−	+/−	−/−	−/−
Patient 2	+/−	+/−	+/−	+/−	+/−
Patient 3	+/−[a]	+/−	+/−	+/−	+/−
Father	+/−	+/−	−/−[b]	+/−	+/−
Sister	+/−	+/−	−/−	+/−	+/−

Nucleotide and amino acid positions are according to the published sequences.[7] E1, -2, -4, and -5 represent exon locations.
[a]One allele contains nucleotide substitution; the other allele is normal.
[b]Both alleles are normal.

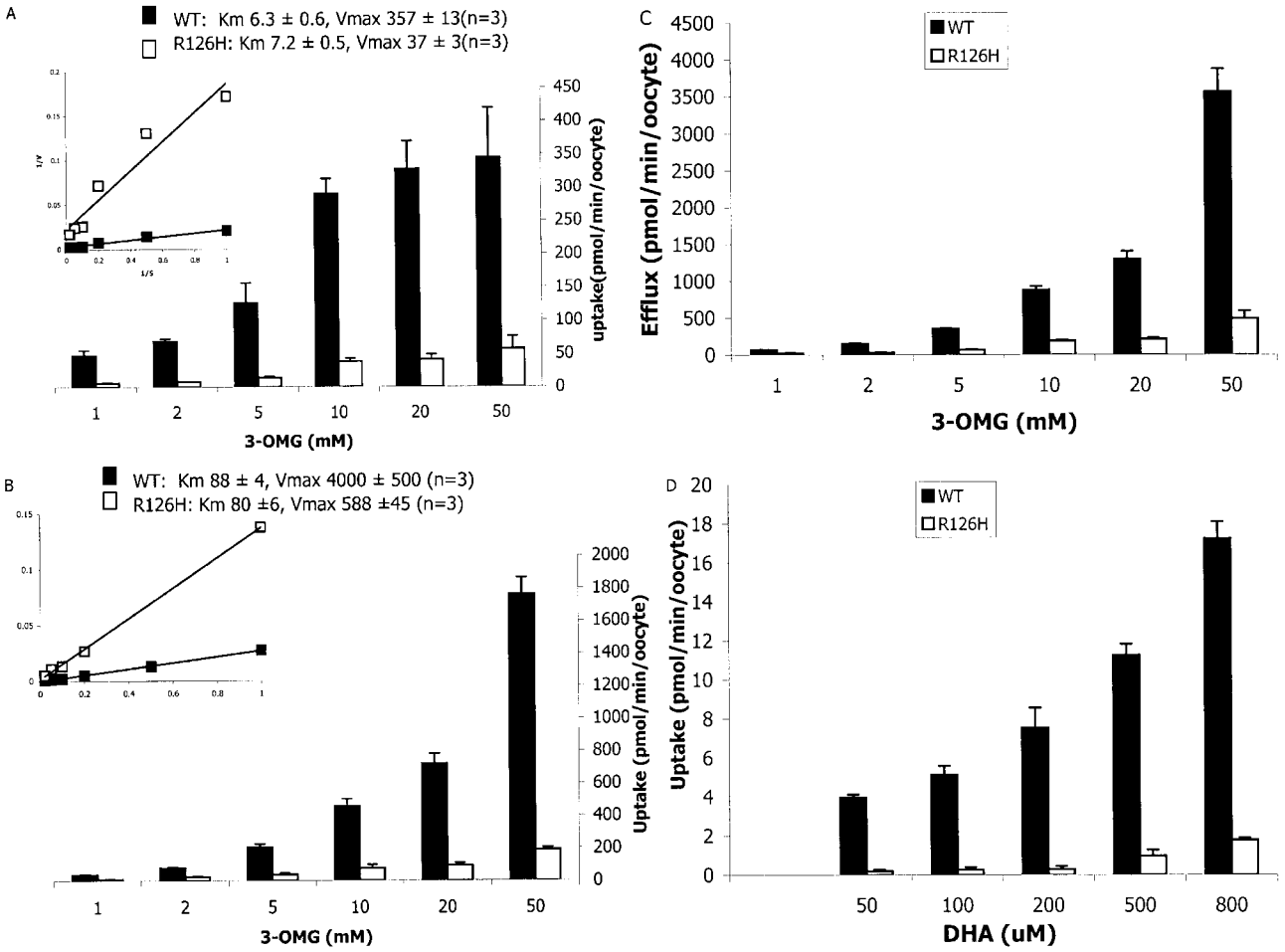


Fig 2. Transport assays in X. laevis *oocytes. Stage V oocytes were injected with water or cRNA encoding wild-type Glut-1 or the mutant R126H. Three days after injection, transport assays were performed under zero-trans influx, equilibrium exchange, and zero-trans efflux conditions for 3-O-methyl-D-glucose (3-OMG), and under zero-trans influx conditions for dehydroascorbic acid (DHA), as described. Uptake values from oocytes injected with water were subtracted from all groups. Values represent mean ± standard error of uptake rates in five oocytes. Results are representative of three independent experiments. (A) 3-OMG zero-trans influx versus concentration; inset is an analysis of 3-OMG uptake kinetics. (B) 3-OMG equilibrium exchange influx versus concentration; inset is an analysis of 3-OMG uptake kinetics. (C) 3-OMG zero-trans efflux versus concentration. (D) DHA zero-trans influx versus concentration.*

concentration curves. The K_i value was calculated from the equation $\upsilon_0/\upsilon = 1 + [I]/K_i$, where [I] is the ethylidene glucose concentration, and υ_0/υ is the ratio of uptake velocity in the absence of inhibitor relative to the uptake at each inhibitor concentration. The K_i for the inhibition of transport activity was 30.8 ± 0.6mM (n = 3) for wild type Glut-1 and 6.5 ± 0.4mM (n = 3) for mutant Glut-1.

Discussion

The 2 probands had received medical care since their first year of life, when they presented with delayed neurological development and seizures. Over the years, they developed a chronic encephalopathy. In both brothers, head growth was normal in infancy, but the older brother (Patient 1) showed deceleration in early childhood. Also, this patient was more neurologically disabled than Patient 2 or Patient 3. We were impressed with the correlations between fasting and neurological deterioration on the one hand, and carbohydrate intake and neurological improvement on the other. Other family members were similarly affected over 3 successive generations, suggesting an autosomal dominant pattern of inheritance (see Fig 1). Antiepileptic drugs were ineffective at controlling seizures. In fact, Glut-1 DS patients are often treated with phenobarbital for infantile-onset seizures, and worsening of seizures has occurred in this patient population. In vitro studies have shown that phenobarbital inhibits Glut-1 facilitated transport of glucose and DHA,[33] and we recently extended these observations to caffeine and theophylline.[34] Neurological worsening with coffee by

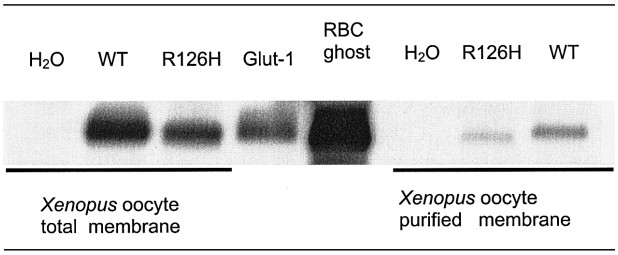


Fig 3. Western blot analysis of total membrane and purified plasma membrane from X. laevis *oocytes. Stage V oocytes were injected with water or cRNA encoding wild-type Glut1 or the mutant R126H. Total membranes and purified plasma membrane were prepared from oocytes 3 days later. Ten micrograms of each sample were subjected to Western blot analysis, as described. Purified Glut-1 (100ng; gift from Dr M. Mueckler, Washington University, St. Louis, MO) and 10μg of red blood cell ghost were run on the same gel as quantitative standards.*

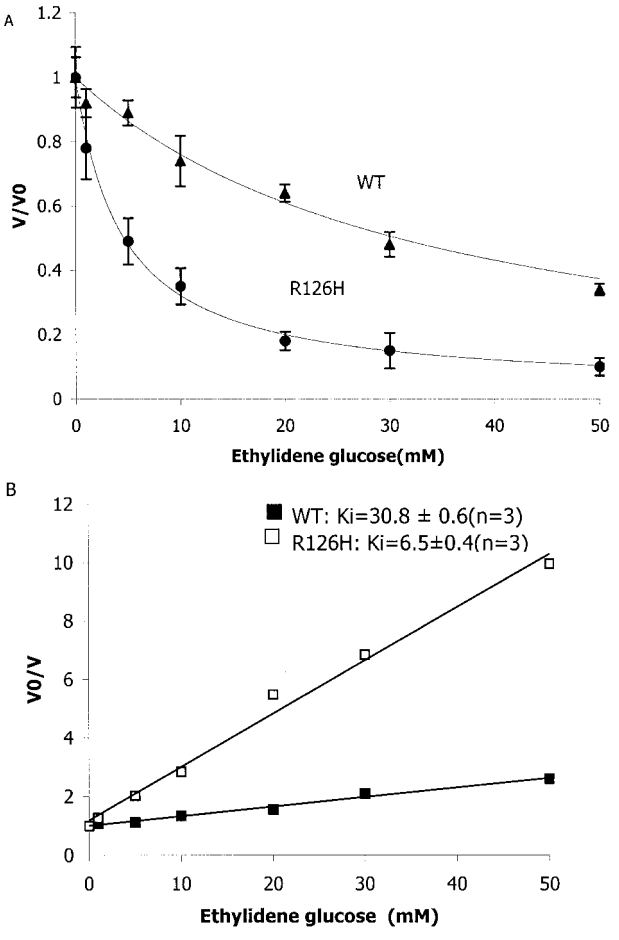
Patient 3 is particularly noteworthy in this regard. This anecdote reinforces our concern that Glut-1 DS patients are vulnerable to Glut-1 inhibitors, worsening clinically.

The Glut-1 immunoreactivity in the erythrocyte membranes was similar to the control activity, indicating that R126H mutant Glut-1 was synthesized normally and incorporated into the plasma membrane. Our previous report of a Glut-1 DS patient with a different heterozygous missense mutation (T310I) demonstrated similar immunoreactive Glut-1 protein expression in erythrocyte membranes.[23] These observations were supported by confocal immunofluorescence microscopy studies on the *X. laevis* oocyte sections, documenting incorporation of mutant Glut-1 into the membrane.

We identified the heterozygous R126H missense mutation in the 2 affected brothers and the mother. The father and the sister were normal at this position. Interestingly, the most severely affected brother (Patient 1) did not carry the heterozygous silent substitutions (GCT→GCC at 224, TGC→TGT at 578, CCG→CCA at 767) and deletion (–173delA) found in his younger brother (Patient 2), mother (Patient 3), father, and sister (see Table 2). This observation suggests that polymorphisms may mitigate the pathogenicity of the missense mutation. Further studies are in progress, including examination of the allelic locations of the mutations, to address this speculation.

Arginine-126 is the last amino acid in the second extracellular loop connecting transmembrane domains 3 and 4 (Fig 5). It is conserved among all known sugar transporters from different species, such as rat, rabbit, bovine, mouse, chick, sheep, and pig. Mutagenesis studies in *X. laevis* oocytes expressing mutant R126H Glut-1 showed a significant decrease in the transport of 3-OMG in zero-trans influx, zero-trans efflux, and equilibrium exchange influx conditions relative to the wild type Glut-1 kinetics. These findings confirm the pathogenicity of this novel missense mutation. The measured transport activities are a function of both the intrinsic catalytic activity and the plasma membrane content of wild type and mutant Glut-1. The expression ratio of mutant R126H Glut-1 in the *X. laevis* oocyte membrane is similar to that of wild type Glut-1, as shown by Western blot (see Fig 3) and confocal immunofluorescent microscopy. These results militate against decreased incorporation into the plasma membrane as a cause of the reduced transport. The normalized K_m of R126H mutant Glut-1 in the zero-trans influx and equilibrium exchange influx stud-

Fig 4. Ethylidene glucose inhibition of 3-OMG uptake. Stage V oocytes were injected with water or cRNA encoding wild-type Glut-1 or the mutant R126H. Ethylidene glucose inhibition of 3-OMG uptake of wild type and R126H mutant Glut-1-expressing oocytes was performed under zero-trans conditions. (A) Fractional uptake rates from one of three independent experiments are shown. Lines represent best fits of the data following the equation $v/v_o = K_i/(K_i + I)$, *where* v/v_o *is the inhibited/noninhibited uptake rates and I is the inhibitor concentration. (B) Data from the same experiment are here fitted to straight lines representing the equation* $v_o/v = 1 + I/K_i$.



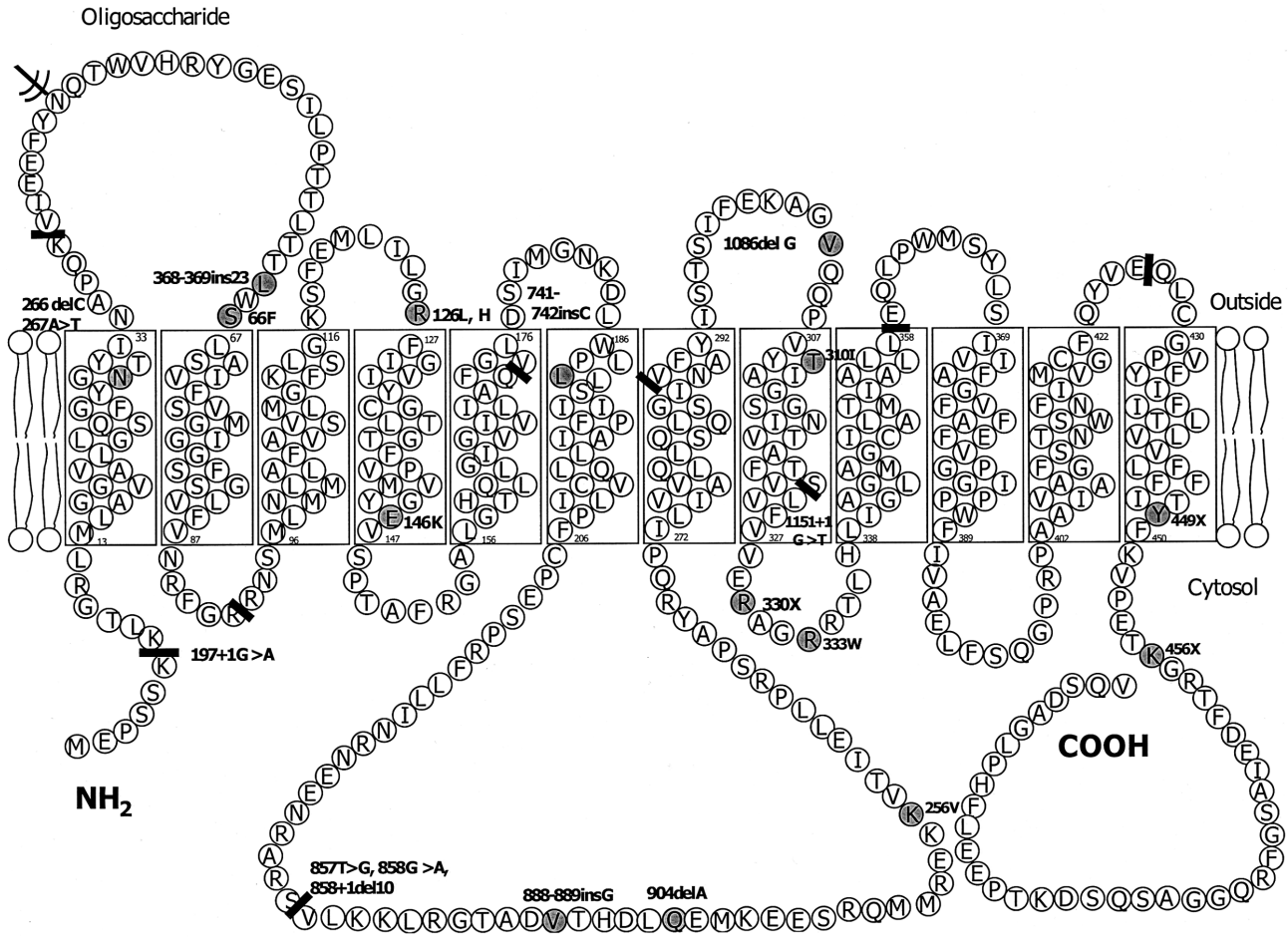


Fig 5. Summary of GLUT-1 *mutations in Glut-1 deficiency syndrome. The 17 identified mutations reported*[22–24] *and the R126H mutation identified in this family are shown in the predicted model of Glut-1 in the plasma membrane; the 3 hemizygous patients are not represented. The 12 transmembrane domains are shown in boxes.* Solid bars *indicate the locations of introns in the* GLUT-1 *gene.*

ies was similar to that of wild type Glut-1, but the normalized V_{max} of mutant R126H Glut-1 was significantly lower than that of wild type Glut-1. This reduction in transport activity cannot be due to decreased affinity of the transported substrate, because the K_m values were similar. Additionally, substrate binding and dissociation are not thought to be rate-limiting for transport.[35] Thus, it is likely that the R126H mutation affects one or more steps in the transport mechanism following substrate binding. Ethylidene glucose inhibition of 3-OMG uptake showed a greater than fourfold increase in the apparent affinity of ethylidene glucose for the outer ligand binding sites of the mutant R126H protein (see Fig 4). This increased affinity was not reflected in a K_m change for a transported substrate, 3-OMG. These data, taken together, indicate that R126 is involved in equilibrium binding of ethylidene glucose to the glucose transporter.

Vitamin C also enters the brain as DHA through Glut-1 at the blood–brain barrier. After entering the brain, DHA is rapidly reduced and trapped as ascorbic acid.[16] This kinetic sequence establishes a gradient for vitamin C between blood and brain. The vitamin C concentration in the human brain is 10 times higher than the serum concentration.[36] Recently, a Na^+-dependent L-ascorbic acid transporter (SVCT2) was identified in the rat brain,[37] but no such active transporters have been identified in the human brain. Vitamin C is a cofactor for dopamine β-hydroxylase and, thus, is involved in the biosynthesis of catecholamines. Vitamin C can also inhibit the peroxidation of membrane phospholipids and act as a scavenger of free radicals in the brain.[38,39] Zero-trans influx studies with DHA showed a significant decrease in the transport activity by the R126H mutant Glut-1 expressed in *X. laevis* oocytes (see Fig 2D). This is consistent with the finding of reduced DHA uptake into erythrocytes isolated from Glut-1 DS patients.[40] We have speculated previously that impaired DHA transport into brain

may contribute to the pathophysiology of this clinical syndrome.[40]

After a few weeks of the ketogenic diet, both brothers improved in their general motor and mental capacities. The younger, less affected brother showed more improvement. Both had significant reduction in seizure frequency, permitting discontinuation of antiepileptic drugs. For reasons stated earlier, we believe that the net clinical improvement is the result of the ketogenic diet and the discontinuation of phenobarbital. The mother, Patient 3, was not able to comply with the ketogenic diet regimen.

These new observations extend the clinical spectrum of Glut-1 DS. In contrast to previously reported cases, none of our patients developed absolute microcephaly. However, Patient 1 did suffer deceleration of head growth in early childhood. This clinical observation may correlate with the relative pathogenicity of the missense mutation. Erythrocyte glucose uptake studies and clinical improvement after carbohydrate loading also correlate with a milder clinical phenotype. Patient 3, to our knowledge the first known adult female with Glut-1 DS, is capable of leading an independent life and caring for 3 children. Her 2 boys are more disabled than she. We speculated that the Glut-1 defect may be potentiated in the affected fetus when the mother also is affected. Transport of glucose in the placenta also is dependent on Glut-1.[41,42] Glucose in the maternal bloodstream passes the fetal apical and basal microvillous plasma membrane through Glut-1.[41,42] Glucose then crosses the endothelial cell facilitated by Glut-1 to enter the blood vessels in the villous core.[41,42] The Glut-1 expressed on syncytiotrophoblasts and the endothelial cell membrane is derived from the fetus. The Glut-1 defect may further decrease the glucose level in the fetal blood. This reasoning, however, suggests that all affected fetuses are equally vulnerable regardless of whether the Glut-1 mutation affects the mother or the father. Of interest, Klepper and colleagues[27] reported a second Glut-1 DS family with autosomal dominant transmission, but in this family, the condition was transmitted from the father to 2 daughters.[27] More studies using a transgenic rodent model for Glut-1 DS will be necessary to pursue this speculation.

In summary, Glut-1 DS is a clinically identifiable condition. Onset of symptoms within the first year of life, with delayed neurological development and seizures and fluctuation of neurological symptoms related to fasting conditions, are highly suggestive of this novel clinical condition. The diagnostic hallmark is hypoglycorrhachia with absolute CSF glucose values less than 40mg/dl. Studies of erythrocyte 3-OMG uptake and demonstration of heterozygous *GLUT-1* mutations confirm the diagnosis. Barbiturates and methylxanthines should be avoided, and a ketogenic diet should be followed at least during the formative period of early brain growth and development. These studies document Glut-1 DS as one biochemical determinant of familial epilepsy, and emphasize the importance of a diagnostic lumbar puncture in the evaluation of patients with unexplained neurological disturbances.

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