

## BRIEF REPORT

## Congenital Glutamine Deficiency with Glutamine Synthetase Mutations

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## SUMMARY

Glutamine synthetase plays a major role in ammonia detoxification, interorgan nitrogen flux, acid–base homeostasis, and cell signaling. We report on two unrelated newborns who had congenital human glutamine synthetase deficiency with severe brain malformations resulting in multiorgan failure and neonatal death. Glutamine was largely absent from their serum, urine, and cerebrospinal fluid. Each infant had a homozygous mutation in the glutamine synthetase gene (R324C and R341C). Studies that used immortalized lymphocytes expressing R324C glutamine synthetase (R324C-GS) and COS7 cells expressing R341C-GS suggest that these mutations are associated with reduced glutamine synthetase activity.

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**G**LUTAMINE SYNTHETASE (GLUTAMATE AMMONIA LIGASE, EC 6.3.1.2) CATALYZES the conversion of glutamate and ammonia to glutamine and is important in ammonia detoxification, interorgan nitrogen flux, and acid–base regulation.<sup>1</sup> High glutamine synthetase activity is found in the human liver, brain, and muscle.<sup>1-4</sup> Human glutamine synthetase consists of eight identical subunits, each containing an active site for the binding of glutamate, ammonia, and ATP.<sup>5</sup> The glutamine synthetase gene has been localized to the region of chromosome 1q31,<sup>6</sup> although a number of genes and pseudogenes have been reported.<sup>7-9</sup>

Diminished glutamine synthetase expression or a secondary deficiency of glutamine synthetase has been described but is rare.<sup>10-13</sup> We describe two unrelated patients with congenital systemic glutamine synthetase deficiency due to mutations in the glutamine synthetase gene.

## METHODS

## PATIENT 1

Patient 1, a boy, was the second child of consanguineous Turkish parents. He was resuscitated at birth and was found to be neurologically compromised, with marked flaccidity, and also to have cardiac insufficiency. He died at two days of life (Table 1).

Cerebral magnetic resonance imaging (MRI) showed a markedly immature brain with hyperintensity of the white matter, enlarged lateral ventricles, and almost complete agyria. Paraventricular cysts, a small, smooth cerebellum, and large pericerebral spaces were present. On postmortem examination the brain weighed only 202 g (335 g expected for gestational age), but no visceral malformations were evident.

**Table 1. Clinical Characteristics and Biochemical Values of the Two Patients and Values of the Parents.\***

Variable	Patient 1	Patient 2
Findings on prenatal ultrasonography	Micromelia, polyhydramnios, large lateral brain ventricles with a left frontal paraventricular cyst	Dilatation of cerebral posterior fossa
Characteristics at birth		
Birth	Spontaneously at 35½ wk	Spontaneously at 38⅔ wk
Weight	2220 g (10th percentile)	2460 g (3rd–10th percentile)
Length	44 cm (3rd–10th percentile)	42 cm (<3rd percentile)
Head circumference	34 cm (75th–90th percentile)	28.5 cm (<3rd percentile)
Initial presentation and clinical course	Respiratory insufficiency, bradycardia, marked axial hypotonia, no spontaneous movements or responsiveness, no alertness, intermittent hyperextension; death on day 2	Generalized muscular hypotonia, respiratory insufficiency, enteropathy with voluminous diarrhea, necrolytic migratory erythema of the skin, ascites, renal failure, pulmonary edema; death in wk 4
Dysmorphic features	Shortness of limbs, flexion contractures at elbows and knees, camptodactyly, ulnar deviation in hands, flat nasal root, short nose, anteverted nares, thin lips, low-set ears	Broad nasal root, low-set ears
Findings on electroencephalography	Outbursts of theta waves, generalized seizures	Multifocal seizures
Glutamine		
Serum	2 µmol/liter (normal range, 433–619 µmol/liter)	6 µmol/liter (normal range, 300–800 µmol/liter)
Urine	Not detectable (normal range, 52–205 mmol/mol creatinine)	8 µmol/g creatinine (normal range, 640–3230 µmol/g)
Cerebrospinal fluid	11 µmol/liter (normal range, 352–885 µmol/liter)	12 µmol/liter (normal range, 520–1280 µmol/liter)
Glutamate		
Serum	45 µmol/liter (normal range, 36–82 µmol/liter)	80 µmol/liter (normal range, 70–220 µmol/liter)
Urine	2 mmol/mol creatinine (normal range, 0–30 mmol/mol)	34 µmol/g creatinine (normal range, 0–250 µmol/g)
Cerebrospinal fluid	Not detectable (normal range, 1–48 µmol/liter)	2 µmol/liter (normal range, 2–51 µmol/liter)
Parental glutamine values		
Serum		
Mother	417 µmol/liter (normal range, 524–642 µmol/liter)	332 µmol/liter (normal range, 300–800 µmol/liter)
Father	500 µmol/liter (normal range, 524–642 µmol/liter)	481 µmol/liter (normal range, 300–800 µmol/liter)
Urine		
Mother		333 µmol/g creatinine (normal range, 235–2010 µmol/g)
Father		404 µmol/g creatinine (normal range, 235–2010 µmol/g)
Parental glutamate values		
Serum		
Mother	20 µmol/liter (normal range, 17–61 µmol/liter)	91 µmol/liter (normal range, 70–200 µmol/liter)
Father	61 µmol/liter (normal range, 17–61 µmol/liter)	111 µmol/liter (normal range, 70–200 µmol/liter)
Urine		
Mother		17 µmol/g creatinine (normal range, 1–110 µmol/g)
Father		17 µmol/g creatinine (normal range, 1–110 µmol/g)

\* Urine samples were analyzed by two different laboratories, which had separate ranges of normal values. Urine samples were not obtained from the parents of Patient 1.

The infant's skin fibroblasts failed to grow, but peripheral blood lymphocytes from the patient and his parents were immortalized successfully by transfection of the Epstein–Barr virus. Two years before his birth, his mother had had a pregnancy terminated at 34 weeks because ultrasonography of the fetal brain revealed abnormal gyrations with mild dilatation of the ventricles suggesting lissencephaly. Micromelia was also evident. However, fluorescence in situ hybridization (FISH) analysis of the region 17p13.3 on amniotic cells ruled out a deletion at the LIS1 locus.

#### PATIENT 2

Patient 2, a girl, was the third child of consanguineous Turkish parents. On the first day of life, convulsions and respiratory failure required intubation and ventilation. During the first weeks of life, the infant had voluminous yellowish stools and progressive weight loss, despite enteral feeding. After two weeks, a generalized blistering erythematous rash developed that on histologic examination supported a diagnosis of epidermal necrolysis. Brain MRI showed markedly attenuated gyri and subependymal cysts. She died during the fourth week of life from multiorgan failure. The only material available from the patient post mortem was her DNA, since her skin fibroblasts failed to grow. However, paternal skin fibroblasts were cultured successfully.

Glutamine was largely absent in the serum from both patients, whereas the glutamate levels were normal. The levels of both glutamine and glutamate were normal in the parents, for whom serum samples, urine samples, or both, were investigated after written informed consent was obtained (Table 1).

#### ELUCIDATION OF THE STRUCTURE OF THE GLUTAMINE SYNTHETASE GENE

A search was performed with the glutamine synthetase transcript sequence (GenBank accession number NM\_002065) and data derived from the Human Genome Project with the use of the Basic Local Alignment Search Tool (BLAST) algorithm ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Sequence alignments permitted the elucidation of the genomic structure of glutamine synthetase, which was confirmed by the sequencing of human liver–derived complementary DNA (cDNA) (Marathon Ready, Clontech) and DNA obtained from healthy control subjects. A GenBank search with the elucidated entire glutamine

synthetase messenger RNA (mRNA) sequence was performed with the use of the nucleotide–nucleotide BLAST as well as the single-nucleotide polymorphism BLAST.

For clarification of the 5' region, 5' rapid amplification of cDNA ends (5' RACE) experiments were performed with the use of glutamine synthetase–specific primers according to the manufacturer's instructions (Boehringer).

#### MUTATION ANALYSIS

DNA was isolated from peripheral blood lymphocytes by standard methods. Polymerase-chain-reaction (PCR) amplification of the glutamine synthetase gene was performed exon by exon with the use of oligonucleotides designed for the flanking intronic regions of all coding exons. Direct sequencing of PCR products was performed with the use of a DNA sequencer (ABI Prism 3700, Applied Biosystems).

To rule out the possibility that the findings represented a common polymorphism, the frequency of the sequence alterations detected was investigated in 50 healthy white control subjects, most of whom were German but some of whom were from other countries in Europe (100 alleles), and in 34 healthy white Turkish controls (68 alleles). Race was determined by the investigators. All subjects provided written informed consent. For the mutation R324C, the enzyme BfrBI (New England Biolabs) was used to digest DNA at the site of the mutation. For the mutation R341C, allele-specific amplification with a primer carrying parts of the restriction site for ClaI (New England Biolabs) permitted the detection of mutated alleles.

Investigations of material obtained from the patients and their parents were performed after written informed consent was obtained from the parents. Healthy controls provided written informed consent for the use of DNA samples rendered anonymous for the analysis of polymorphisms. The study was approved by the institutional review board of the University of Münster.

#### ALIGNMENT OF MUTATIONS

Sequence alignment with eukaryotic and prokaryotic species was performed with the use of the GenBank BLAST algorithm in order to estimate the degree of evolutionary conservation. For estimation of the functional relevance of the mutations found, a model of the human enzyme was constructed on the basis of the x-ray structure of glutamine

synthetase from *Salmonella typhimurium* (protein data bank code 1FPY).<sup>14</sup>

#### CLONING AND TRANSFECTION OF WILD-TYPE AND MUTATED GLUTAMINE SYNTHETASE PLASMIDS

As a template for PCR amplification of the entire glutamine synthetase cDNA (1122 bp), cDNA derived from human liver (Marathon-Ready, Clontech) was used. PCR amplification was performed with glutamine synthetase-specific primers with the use of plaque-forming unit-DNA-polymerase (Clontech) at an annealing temperature of 67°C. The product was purified (PCR Purification Kit, Qiagen) and directly ligated into the previously BamHI- and XbaI-digested eukaryotic expression vector pcDNA3.1HisA (Invitrogen). The ligation product was transformed into a Top10F' *Escherichia coli* strain. Correct plasmids were selected by sequencing and were named pcDNA3.1GS-WT.

Site-directed in vitro mutagenesis with the use of the wild-type plasmid as a target was performed for introduction of the alteration c.1021C>T into the expression plasmid, thereafter coding for R341C glutamine synthetase (R341C-GS). Methylation and mutagenesis reactions were performed with the use of the GeneTailor Site-Directed Mutagenesis System (Invitrogen). In brief, methylated plasmids (20 ng) were used for the mutagenesis reaction catalyzed by Platinum Taq DNA Polymerase High Fidelity. DH5 $\alpha$ T1 *E. coli* were transformed with the mutagenesis product, and the resultant plasmid was control sequenced and was named pcDNA3.1GS-Mut.

COS7 cells (i.e., a line of African green-monkey kidney cells transformed by simian virus [SV] 40) were transfected with the plasmids pcDNA3.1GS-WT and pcDNA3.1GS-Mut with the use of cationic lipids (in a molar lipid-to-DNA ratio of 2:1) (TransFast Transfection Reagent, Promega). Successfully transfected cells were selected by adding G418 (Geneticin, GIBCO) to the culture medium (200  $\mu$ g per milliliter). GS-WT, GS-Mut, and a non-transfected cell line serving as a control were grown for 14 to 21 days at 37°C in Dulbecco's modified Eagle's medium with 10 percent fetal-calf serum (Invitrogen) in a humidified atmosphere with 5 percent carbon dioxide in six 75-cm<sup>2</sup> flasks (Falcon).

#### ANALYSIS OF GLUTAMINE SYNTHETASE EXPRESSION

Cells were harvested in buffer containing 10 mM TRIS hydrochloride (pH 7.4), 1 percent Triton X-100,

150 mM sodium chloride, 1 mM EDTA, 1 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N*-tetraacetic acid, 1 mM sodium vanadate oxide, 20 mM  $\beta$ -glycerophosphate, 20 mM sodium fluoride, 10 mM sodium diphosphate, and a protease-inhibitor cocktail (1 tablet per 50 ml) (Boehringer). The protein content was estimated<sup>15</sup> with the use of a reagent from BioRad. Proteins were analyzed by Western blotting with the use of 10 percent sodium dodecyl sulfate-polyacrylamide gels and a semi-dry transfer apparatus (Amersham Pharmacia). Blots were probed with antisera against glutamine synthetase (1:5000 solution) (polyclonal antibody, Santa Cruz Biotechnology; monoclonal antibodies, Transduction Laboratories and Chemicon) and against His-Tag (1:5000 solution) (Invitrogen) for two hours. After washing and incubation with horseradish-peroxidase-coupled antimouse-IgG or antigoat-IgG antibody diluted in a 1:10,000 solution at 4°C for two hours, the blots were developed with the use of Western-Lightning Chemiluminescence Reagent Plus (PerkinElmer).

#### ANALYSIS OF GLUTAMINE SYNTHETASE ACTIVITY

The glutamine synthetase-catalyzed formation of  $\gamma$ -glutamylhydroxamate from glutamine and hydroxylamine was measured.<sup>16</sup> Aliquots of cell lysates containing equal amounts of protein were incubated with 60 mM of L-glutamine, 15 mM of hydroxylamine-hydrochloride, 20 mM sodium arsenite, 0.4 mM adenine diphosphate, 3 mM magnesium chloride, and 60 mM imidazole-hydrochloride buffer (pH 6.8) in a final volume of 1 ml at 37°C. The reaction was terminated by the addition of 1 ml of stop solution containing 0.2 M of trichloroacetic acid, 0.67 M hydrochloride, and 0.37 M iron chloride. The solution was cleared of protein by centrifugation (14,000 $\times$ g) at 4°C and glutamylhydroxamate measured in the supernatant at 500 nm.

#### STATISTICAL ANALYSIS

Results ( $\pm$ SE) were compared with the use of Student's t-test. A difference was considered statistically significant if the P value was less than 0.05.

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## RESULTS

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#### GENE STRUCTURE

The GenBank-based search revealed the putative genomic structure of glutamine synthetase. After sequencing, the confirmed sequences of

glutamine synthetase mRNA and DNA were deposited (GenBank accession numbers AY486122 and AY486123, respectively). The glutamine synthetase mRNA consists of 1122 bp comprising 6 exons encoding a 374-amino-acid polypeptide with an estimated molecular mass of 42 kD.

#### MUTATION ANALYSIS

Sequencing of PCR fragments in both directions and restriction digestion showed different mutations in each of the two families: c.970C>T (R324C) in the family of Patient 1 and c.1021C>T (R341C) in the family of Patient 2 in exon 6 of the glutamine synthetase gene (Fig. 1). The mutations were ruled out as common polymorphisms, since none of the

160 control alleles (including 60 alleles from Turkish persons) showed these sequence alterations.

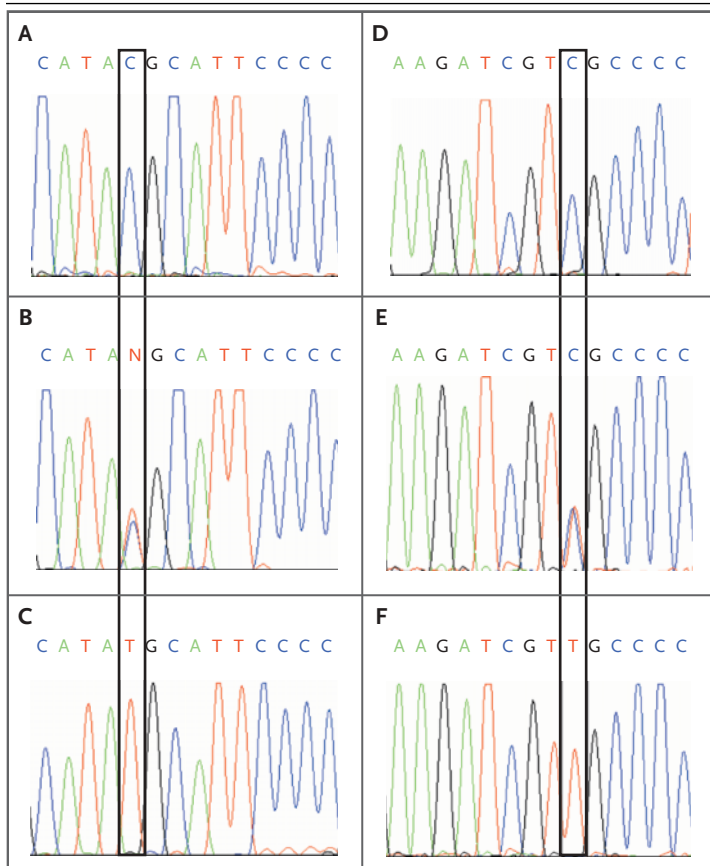
#### ALIGNMENT OF MUTATIONS

Sequence alignment showed R324 (human) to be strictly conserved in prokaryotic and eukaryotic glutamine synthetase and, likewise, that R341 was highly conserved. According to a model of human glutamine synthetase based on the x-ray structure of glutamine synthetase of *S. typhimurium*, R324 is part of the glutamate binding pocket, which stabilizes the  $\gamma$ -phosphate 1 moiety of ATP and is part of a sequence motif (ASIRIP). The arginine residue at codon R324 appears to be strictly conserved in prokaryotic and eukaryotic species. The arginine residue at codon R341 is in close proximity to highly conserved residues that are part of the active site of glutamine synthetase, forming a pocket for ATP and glutamate.

#### ANALYSIS OF GLUTAMINE SYNTHETASE EXPRESSION AND ACTIVITY

In immortalized lymphocytes from Patient 1, glutamine synthetase activity was reduced to about 12 percent of that found in immortalized lymphocytes from wild-type controls. In the cells of the parents of Patient 1, glutamine synthetase activity was reduced to about 71 percent (in the father) and 49 percent (in the mother) (Fig. 2A, gray bars). Increased glutamine synthetase expression was observed in the lymphocytes from Patient 1 and his mother. The normalization of glutamine synthetase activity to the level of glutamine synthetase expression found in the Western blotting test (Fig. 2A, black bars) indicates that increased glutamine synthetase expression may, in part, compensate for the loss of specific glutamine synthetase activity.

In cell lysates from transfected COS7 cells, the polyclonal anti-glutamine synthetase antibody and the anti-His-Tag antibody recognized both wild-type glutamine synthetase and R341C glutamine synthetase (R341C-GS). Two monoclonal anti-glutamine synthetase antibodies were ineffective in detecting R341C-GS but recognized wild-type glutamine synthetase, supporting the view that this mutation induces a structural change. As compared with wild-type glutamine synthetase, R341C-GS was less efficiently expressed in COS7 cells (Fig. 2B). Glutamine synthetase activity normalized to the level of His-Tag immunoreactivity was significantly reduced in COS7 cells expressing R341C-GS (Fig. 2C). In fibroblasts from the father of Patient 2,



**Figure 1.** Alignments of Glutamine Synthetase Nucleotides.

The alignments of glutamine synthetase nucleotides c.966 to c.979 are shown in Panels A, B, and C and those of glutamine synthetase nucleotides c.1013 to c.1026 are shown in Panels D, E, and F, with the respective wild-type sequence shown in Panels A and D. The sequence found in the father of Patient 1 is shown in Panel B and that of the father of Patient 2 in Panel E. The sequence of Patient 1 is shown in Panel C and that of Patient 2 in Panel F, illustrating the mutations c.970C>T in Family 1 and c.1021C>T in Family 2.

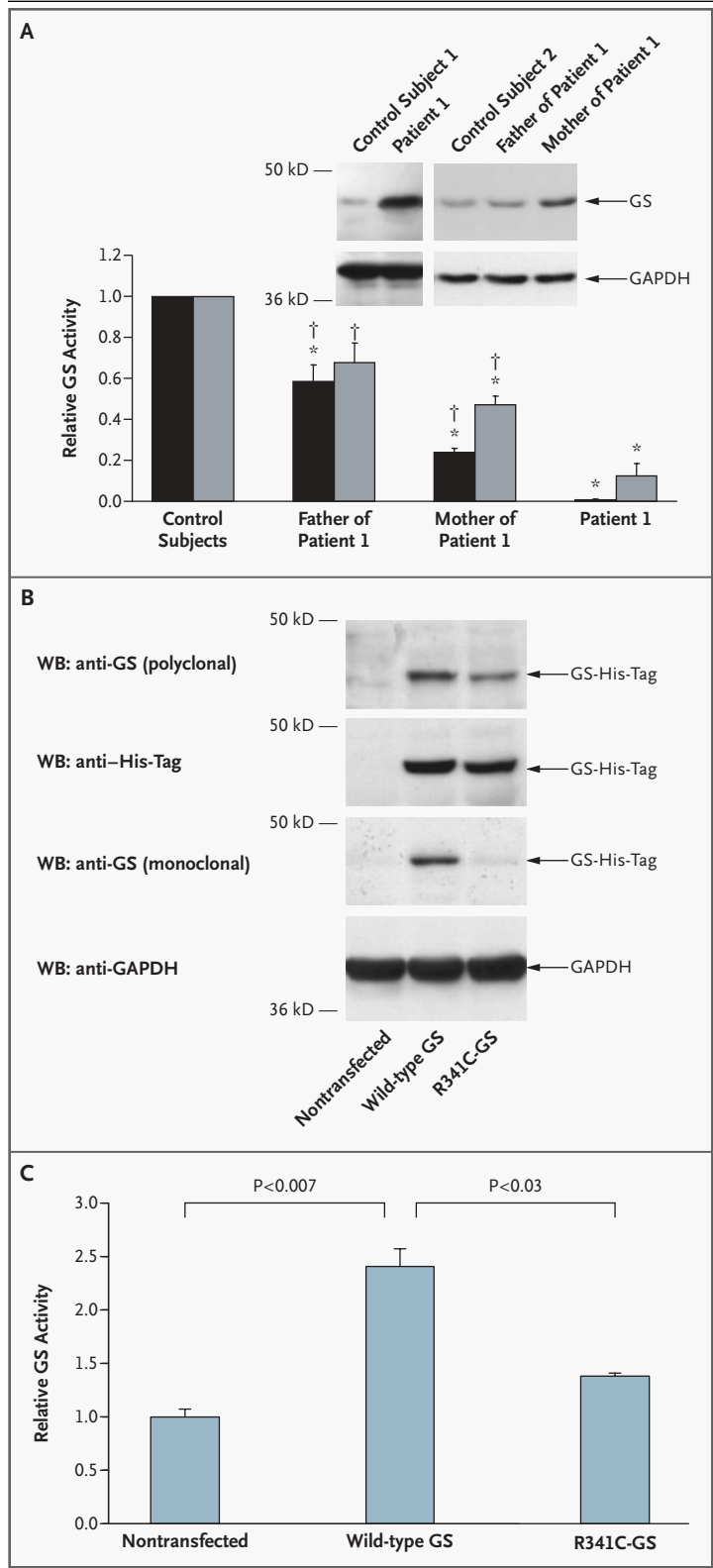
**Figure 2. Glutamine Synthetase Expression and Activity.**

Panel A shows immortalized B lymphocytes from Patient 1, the patient's heterozygous parents, and two wild-type controls that were analyzed for the expression of glutamine synthetase (GS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and for GS activity. The differences in the levels of GS expression were quantified by densitometry. The GS activity measured in the two wild-type controls was set to 1. The relative GS activity in equal amounts of total protein (blue bars) was normalized to equal levels of GS expression (black bars). The increased GS expression found in Patient 1 and in his mother suggests partial compensation for a loss of specific GS activity. The samples from the two healthy controls were each measured in duplicate, and the levels of GS activity were similar ( $4.5 \mu\text{mol}$  per gram of protein per hour and  $5.0 \mu\text{mol}$  per gram of protein per hour). Three aliquots from Patient 1 and from each of his parents were measured. The asterisks denote a difference ( $P < 0.05$ ) in GS activity between the samples of Patient 1 and his parents from the wild-type controls, and the daggers denote a difference ( $P < 0.05$ ) in GS activity between the samples from Patient 1 and his parents. Panel B shows data from COS7 cells transfected with plasmids that encode either tagged wild-type GS or R341C-GS and nontransfected cells that were analyzed for GS expression with the use of an anti-His-Tag antibody and the polyclonal or monoclonal antibodies against GS. Although the polyclonal antibody recognized both the wild-type GS and the mutant GS, the monoclonal antibodies (Transduction Laboratories and Chemicon [not shown]) recognized wild-type GS but not the mutant GS. As shown in Panel C, for the determination of GS activity, equal amounts of total proteins were assayed in triplicate. The values of His-Tag immunoreactivity were quantified by densitometry and taken into account in the calculation of the GS activity by the normalization of GS activity to equal levels of His-Tag expression. Bars denote SD. WB denotes Western blot.

glutamine synthetase expression was increased. This increase may compensate for the approximately 50 percent drop in specific glutamine synthetase activity found in these cells (data not shown).

#### DISCUSSION

Congenital systemic glutamine deficiency was the main biochemical finding in two neurologically devastated neonates with multisystem disease. The most striking features were marked brain malformations with abnormal gyration and marked white-matter lesions. Substitutions of arginine for cysteine at codons 324 and 341 were detected in Patient 1 and



Patient 2, respectively. Parental heterozygosity suggested an autosomal recessive mode of inheritance and confirmed segregation of the mutations in the two families.

Despite an increase by about a factor of six in R324C-GS protein expression, immortalized lymphocytes from Patient 1 exhibited a considerable loss of glutamine synthetase activity as compared with lymphocytes from his parents and from the controls. As previously suggested,<sup>17</sup> low levels of intracellular glutamine may up-regulate glutamine synthetase expression within a feedback regulatory loop. In the case of heterozygous carriers, this up-regulated expression may help to achieve glutamine synthetase activity sufficient to permit normal cell function. Transfection studies delineated the inactivation of glutamine synthetase as a result of the R341C exchange, supporting the disease-causing role of the mutation in Patient 2.

Glutamine is a major source of energy and is involved in cell proliferation, apoptosis inhibition, and cell signaling.<sup>18-20</sup> Fetal glutamine requirements are very high and depend largely on active glutamine synthesis and the release of glutamine into the fetal circulation by the placenta.<sup>21</sup> Although the levels of serum glutamine in heterozygous carriers were normal, maternal glutamine synthesis may be insufficient to guarantee an adequate supply of glutamine to the fetus. In addition, a loss of

specific glutamine synthetase activity in the placenta derived from the fetus may further limit the glutamine fetal supply. In samples obtained from both Patient 1 and Patient 2 and in a fetal sample from a terminated pregnancy of the mother of Patient 1, fibroblast cultures failed to grow in spite of the presence of extracellular glutamine, pointing to a need for glutamine synthesis in cultures of fibroblasts from such patients. Interestingly, the concentration of glutamate (i.e., the substrate proximal to the metabolic block) was not elevated in either of the two patients, but this lack of elevation might be due to the various other pathways of glutamate catabolism.

In summary, we describe a congenital syndrome that results from systemic glutamine deficiency caused by a defect of the glutamine synthetase gene. Brain malformation was the principal manifestation in these two patients. This report might prompt others to consider this disorder in neonates with similar brain malformations. Obtaining serum glutamine levels in such neonates would be useful in screening for the disorder.

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BRIEF REPORT

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