Chronic non-spherocytic hemolytic anemia associated with severe neurological disease due to γ-glutamylcysteine synthetase deficiency in a patient of Moroccan origin

A previously undescribed mutation of hereditary γ-glutamylcysteine synthetase (GCS) deficiency was found in a 5 year old boy of Moroccan origin. He presented with chronic haemolytic anaemia, delayed psychomotor development and progressive motor sensitive neuropathy of lower extremities. The parents were third degree relatives. The activity of glycolytic enzymes were found to be normal in the propositus, his parents and a sister, but and a complete lack of GSH was found in the propositus. Accordingly, the measurement of de novo GSH synthetic enzymes was undertaken, and severe GCS deficiency was found in the propositus. Both parents and his sister presented GCS activity ranging from 69% to 90% of normal. *GCS* gene sequencing showed that the propositus was homozygous for a 1241C>T mutation in exon 11 and both parents and his sister were heterozygous. This mutation predicts a Pro414Leu amino acid substitution. Even though the homology between GCS and crystallographically solved, functionally related proteins is not very high, a three-dimensional model of GCS was derived using Modeller Software. GCS deficiency is a very rare autosomal recessive disorder reported so far in only 8 unrelated probands with severe haemolytic anaemia. In only 3 of these was the anaemia associated with severe neurological dysfunction. We report here the fourth case of GCS deficiency presenting neuropathy, giving further support to the eventual relationship between this enzymopathy and neurological damage

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Glutathione (L-γ-glutamyl-L-cysteinyl-glycine;GSH) is an important intracellular antioxidant tripeptide necessary for the protection of cells from damage by oxygen intermediates, free radicals, peroxides, and toxins of both endogenous and exogenous origin.[1,2] Two rate-limiting enzymes are involved in *de novo* ATP-dependent biosynthesis of red blood cell GSSG: γ-glutamylcysteine synthetase, (GCS), also known as glutamate-cysteine ligase (GCL) and glutathione synthetase (GS).

GS is a 118 kD homodimer that catalyses the addition of glycine to the cysteine carboxyl group of GGC to form GSSG. Hereditary deficiency of GS is an autosomal recessive disorder that has been reported in 41 unrelated patients. In its severe form it is characterized by haemolytic anaemia, metabolic acidosis with massive urinary excretion of 5-oxoproline (5-oxoprolinuria) and central nervous system damage.[3-5]

GCS is a 104 kD heterodimer that consists of a catalytic (-GCSH) and modifier (-GCSL) subunit and catalyzes the amide linkage between cysteine and the γ-carboxyl group of glutamate to form the dipeptide γ-glutamylcysteine (GGC). Hereditary deficiency of GCS is a very rare autosomal recessive enzymopathy so far reported in only 8 unrelated families.[6-11] The common clinical manifestation of GCS deficiency is recurring bouts of anaemia and jaundice but it has been found to be associated with severe neurological abnormalities in two cases[6,12] and mental retardation in one.[10] In the two most recently reported cases of GCS deficiency[9-11] single-point mutations in the coding sequence of GCS gene have been identified as the underlying molecular cause of the condition.

We report here a novel mutation, a single C>T transversion at cDNA nucleotide 1241 in the γ-GCS gene, found in a patient of Moroccan origin. This is the fourth case of GCS deficiency so far described in which chronic anaemia is associated with both severe neuropathy and mental retardation.

Materials and mathods

Case report. A 5 year old boy of Moroccan origin presented with chronic haemolytic anaemia associated with progressive motor neuropathy of low extremities and psychomotor development. The patient's parents were consanguineous but clinically normal. Neurological impairment had its onset shortly after birth, becoming apparent at 18 months of age, when the child began to walk, partially masked by the normal development of motor skills between the age of 2 to 5 years. After this age the child developed a progressive loss of flexor strength in his legs associated with arreflexia the lower extremities, cavus foot and a peculiar gait in which both legs were stretched and separated. On physical examination, the patient exhibited short stature (<P3), hypertelorism, epicanthus's, dental malposition, achanthosis nigricans, and cutis marmorata. Speech retardation was manifested by the use of mono- or bi-syllabic words only. Magnetic nuclear resonance of brain and electroencephalography were normal and neuro-electrophysiologic studies showed slow peripheral nerve conduction in both legs consistent with a demyelization peripheral neuropathy. No progression of the nerve conduction abnormality has been observed during the past few years. The patient did not have metabolic acidosis and no urinary excretion of 5-oxoproline or aminociduria was detected. Routine haematological studies performed at diagnosis showed a compensated haemolysis with normal haemoglobin concentration (Hb: 110 g/L) and an increased number of reticulocytes (6.56%). Platelet and leukocyte counts were normal and there were no specific abnormalities of red blood cell morphology. Blood biochemistry demonstrated elevated serum lactate dehydrogenase activity and plasma iron and ferritin levels. Serum haptoglobin was markedly decreased. Serum vitamin B12 (cobalamin) and folate concentrations, Coomb's test, RBC osmotic fragility test and acidified serum test were normal. HPCL and heat stability studies for abnormal haemoglobins ruled out the existence of haemoglobinopathy. Finally, ultrasound studies did not reveal any evidence of gallstones.

Red blood cell enzyme activities and GSH. Heparinized blood samples were obtained from the propositus, his parents and a sister. Packed RBCs were freed from leukocytes by using a cellulose column and washed 3 times with 5 volumes of isotonic NaCl solution. After centrifugation at 900g for 5 minutes, the RBCs were diluted 1/20, frozen in liquid nitrogen for 10 minutes and stored at -80°C until study. A panel of 23 RBC enzyme assays was determined using the methods standardized by ICSH.[13] The GSH content of patient's and her family RBCs was measured according to the method described by Beutler.[14] Activity of enzymes involved in the biosynthesis of GSH; glutamylcysteine synthetase (GCS) and glutathione synthetase (GS) were determined according to the method of Ristoff *et al.*[10]

Sequence analysis of GCS gene. Genomic DNA was isolated from peripheral blood leukocytes of the propositus, his parents and a sister by standard methods. Using GenBank sequence NC_000006.8 GI:42406225 sense and anti-sense oligonucleotide primers were designed to PCR amplify each of the 16 exons and their flanking intronic sequences of the gene encoding the catalytic subunit of γ-glutamylcysteine synthetase. The amplified PCR fragments were sequenced on an Applied Biosystems Inc. (Foster City, CA) automatic sequencer using the dideoxy termination method.

Modelling of GCS. The sequence from GCS (NP_001489) was compared with the Protein Data Bank sequences using the program BLAST[15] and Modeller[16] to identify protein templates for building the protein model. No templates were obtained with the whole protein sequence, indicating the very low homology with functionally related solved structures. The PFAM database[17] was queried to look for protein domains and families; a clear correspondence to glutamate-cysteine ligase family was found indicating that the catalytic subunit of GCS was in residues 235 to 609 of the GCS molecule. Accordingly, this portion of the protein will be designated as the GCS domain.

Residues of the GCS domain were considered in the final alignment procedure and a good score was obtained with the crystallographic structure of YbdK, an *E. Coli* carboxylate-amine ligase with a γ-glutamyl cysteine ligase activity (PDB ID 1R8G), with a high identity with our domain.[18] The structural model was derived from the Modeller program[16] and, in order to eliminate unfavorable contacts, minimization of the structure was performed using the Swiss-PdbViewer tutorial.[19] Fold-X software was used[20] for the stability calculations performed on the modeled protein with the mutation of interest (P414L).

Results

Red blood cell enzyme activities. Activities of glycolytic enzymes and enzymes associated with redox cycling studied in the patient and her parents and sister were normal or slightly increased in the propositus, due to the decreased mean erythrocyte age. The patient's RBCs GSH content and that his relatives is shown in Table 1. In the propositus, the RBC GSH was less than 5% of normal. Table 2 summarizes the activities of enzymes involved in the de novo synthesis of GSH; γ-glutamylcysteine synthetase (GCS) and glutathione synthase (GS) in both the propositus and his relatives. Whereas GS activity was normal in all cases, GCS activity was less than 1% of the normal in the patient and between 69% and 90% in her parents and her sister.

Sequencing analysis of GCS gene. The amplified DNA from the whole GCS coding region (16 exons) from the propositus and his mother was sequenced. The propositus was found to be homozygous for a 1241C>T transition in exon 11 and his mother was found to be heterozygous for same mutation. In addition, a number of previously known polymorphisms were identified in the exons. Sequencing the amplified DNA from exon 11 from the father and the sister showed that both were heterozygous for the1241 C>T mutation. The 1241 C>T transition predicts a Pro414Leu amino acid substitution in the protein. This amino acid is conserved between human, mouse, rat, and drosophila.

Modelling of GCS protein. In our model, the wild type GCS, the Pro414 is located at the end of a short helix. This residue is only partially exposed, and establishes close hydrophobic contacts with a LYS352 residue located in the facing helix. The change of residue PRO to LEU would produce unfavorable contacts, mainly for steric reasons, between the new residue and the LYS352. Also, the ΔG value of about 2 Kcal/mol

Table 1. GSH content (mg/dL) of patient's RBCs and his relatives

	GSH	*GSH + APH*
Patient	3.03	0.55
Mother	89.7	67.6
Father	76.5	68
Sister	93.3	64.5
Reference Range	54.7-82.7	42.0-71.1

APH: Acetylphenylhydrazine

Table 2. Activities of enzymes (pkatal/mg Hb) involved in the *de novo* synthesis of glutathione

	Glutathione synthetase (GS)	*Gamma-glutamylcysteine synthetase (GCS)*
Patient	8.5	0.12*
Mother	7.5	14.6
Father	7.4	15.8
Sister	7.3	18.9
Control	7.0	21
Reference Range	3.6-8.6	19.5-23.8

obtained with Fold-X (20) for that mutation, corroborates that it is a non stabilizing change. Our model (Figure 1) shows the archetypical fold of glutamate-cysteine ligase families, characteristic of the α *and* β proteins class.

Discussion

GSH is a tripeptide found in high concentrations in several tissues such as the liver, kidney, brain, muscle and red cell. It plays an important role in biological functions, including synthesis of proteins and DNA, detoxification, and redox reactions. There are five different enzymes involved in GSH metabolism, two related with its synthesis: γ-glutamylcysteine synthetase (GCS) and glutathione synthase (GS), two glutathione redox cycling: glutathione reductase (GR), glutathione peroxidase (GP), and glutathione transferase (GT). Enzyme deficiencies with an autosomal recessive mode of inheritance have been reported in all these enzymes.

Genetically determined deficiencies of enzymes involved in the synthesis of GSH are associated with very low levels (<20%) of GSH in RBCs, haemolytic anaemia and, in some patients, progressive neurological disease. In GSH deficiency due to GS deficiency, several cases have been so far documented existing in two main different clinical forms: chronic haemolytic anaemia alone or associated with neuromuscular and metabolic disorder with massive urinary excretion of 5-oxoproline (oxoprolinuria). In contrast, the GSH deficiency caused by GCS deficiency is extremely rare since only 8 cases have been reported so far. GCS deficiency is also a known cause of chronic haemolytic anaemia but in three out of the eight cases it has been associated with progressive neurological manifestations. The first report of this enzymopathy was provided by Konrad *et al.* in 1972 in two related patients, a brother and a sister, of German ancestry.[6] The same cases were reported by Richards *et al.* in 1974.[7] Both patients showed relatively well compensated anaemia and progressive spinocerebellar degeneration. In both cases the diagnosis was made by the measurement of erythrocyte GSH, which was found to be less than 5 per cent of normal and severely decreased GCS activity (between 2 to 7% of normal) with normal GS activity was found in both patients. The responsible mutation, a 379C>T leading to an Arg127Cys amino acid change, was identified in 2003.[12] After this first description of two cases of GCS deficiency in 1974,[6,7] six new cases have been reported, two unrelated patients by Beutler *et al.* in 1990[8] and in 1999,[10] respectively, two other cases by Hirono *et al.* in 1996[9] and another two by Ristoff *et al.* in 2000.[11] In most of these cases, a moderate to severe haemolytic anaemia appeared during neonatal period or in the early childhood that persisted during all the life or compensated haemolysis with sporadic recurring bouts of anaemia and jaundice. The first two German cases reported[6,7] developed, in addition to haemolytic anaemia, learning disability with dyslexia, severe and progressive ataxia associated to myopathy, thought to be due to spinocerebellar degeneration and psychotic crises, probably related with the ingestion of sulphametoxazol-trimethoprim.[6,7,10]

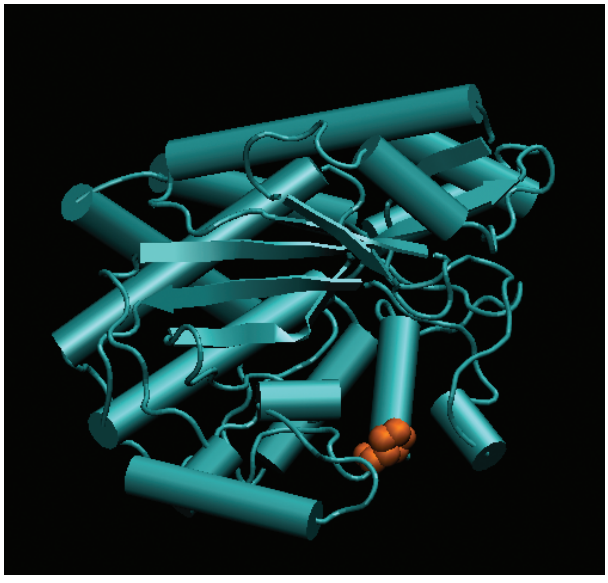

Figure 1. Three-dimensional model of human GSC obtained with the VMD program.[19] Prp 414 is shown in orange as van der Waals representation.This image was made with VMD/NAMD/BioCoRE/JMV/software developed with NIH support by the Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign.

The existence of possible relationship between the GCS deficiency and neurological disease has been matter of discussion for many years. The fact that the majority of the patients with this enzymopathy have been free of neurological findings, cast doubt on the existence of a cause-and-effect relationship.[8] Such a relationship, however, has been well established for other RBC genetic disorders where variants with and without neurological stigmata occur. Examples of this situation include GS deficiency and NADH-diaphorase deficiency (Cytochrome b5 reductase deficiency).

The patient reported here is the fourth case of GCS deficiency where a well compensated haemolytic anaemia is associated with severe neurological disease and mental retardation. This provides further support to the existence of a relationship between this enzymopathy and neurological impairment. As for the other RBC enzyme defects that can also occur with neurological disease, the mechanism of this relationship is probably related to the distribution of the enzyme deficiency. One approach is the genetic characterization of deficient enzymes and the study of mutation positions by molecular modelling. GCS consists of two subunits, a heavy and a light subunit encoded by two different genes GLCLC and GLCLR, respectively. The heavy subunit (GCSh) has catalytic activity and the light unit regulato-

ry functions (GCSl). Up to now, only three mutations have been reported in GCS deficiency, all within the GCSh subunit: 1-1109A>T, with a change of histidine to leucine in position 370 (His370Leu), 2- 473C>T with change of proline to leucine in position 158 (Pro158Leu), and 3- 379C>T with change of arginine to cysteine in position 127 (Arg127Cys). We report here the fourth case with GCS deficiency due to a mutation at the GCSh subunit. Genetic characterization of this case demonstrated homozygosity for the 1241C>T, predicting a Pro-Leu substitution at amino acid 414. Our model of GCS domain indicates a preservation of the archetypical folding of Glutamate-cysteine ligase family, and that the spatial position derived here for the highly conserved Pro414 may explain the destabilizing properties of the mutation.

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References

1. Kosower NS, Kosower ES. Glutathione metabolism and function. Annu Rev Biochem 1983; 52:711-60.
2. Meister A. Metabolism and Function of Glutathione. In: Dolphin D, Avramovich A, Poulson R, eds. Glutathione: Chemical, Biochemical, and Medical Aspects. New York, NY: John Wiley and Sons; 1989; 367-474.
3. Dahl N, Pigg M, Ristoff E, et al. Missense mutations in the human glutathione synthetase gene result in severe metabolic acidosis, 5-oxoprolinuria, hemolytic anemia and neurological dysfunction. Hum Mol Genet 1997; 6:1147-52.
4. Ristoff E, Mayatepek E, Larsson A. Long-term clinical outcome in patients with glutathione synthetase deficiency. J Pediatr 2001; 139:79-84.
5. Corrons JL, Alvarez R, Pujades A, et al. Hereditary non-spherocytic haemolytic anaemia due to red blood cell glutathione synthetase deficiency in four unrelated patients from Spain: clinical and molecular studies. Br J Haematol 2001; 112:475-82.
6. Konrad PN, Richards F, Valentine WN, Paglia DE. -Glutamylcysteine synthetase deficiency. A cause of hereditary hemolytic anemia. N Engl J Med 1972; 286: 557-61.
7. Beutler E, Moroose R, Kramer L, Gelbart T, Forman L. γ-glutamylcysteine synthetase deficiency and hemolytic anemia. Blood 1990; 75:271-3.
8. Hirono A, Iyori H, Sekine I, et al. Three cases of hereditary nonspherocytic hemolytic anemia associated with red blood cell glutathione deficiency. Blood 1996; 87:2071-4.
9. Beutler E, Gelbart T, Kondo T, Matsunaga AT. The molecular basis of a case of γ-glutamylcysteine synthetase deficiency. Blood 1999; 94:2890-4.
10. Ristoff E, Augustson C, Geissler J, et al. A missense mutation in the heavy subunit of γ-glutamylcysteine synthetase gene causes hemolytic anemia. Blood 2000; 95:2193-6.
11. D. Hamilton, J. Hui Wu, M. Alaoui-Jamali, Gerald Batist. A novel missense mutation in the γ-glutamylcysteine synthetase catalytic subunit gene causes both decreased enzymatic activity and glutathione production. Blood 2003; 102:725-30.
12. Richards F, Cooper MR, Pearce LA, Cowan RJ, Spurr CL. Familial spinocerebellar degeneration, hemolytic anemia, and glutathione deficiency. Arch Intern Med 1974; 134:534-7.
13. International Committee for Standardization in Haematology. Recommended methods for red cell enzyme analysis. Br J Haematol 1977;35:331.
14. Beutler E. The glutathione instability of drug sensitive red cells. J Lab Clin Med 1957;49:84.
15. Altschul P, Gish W, Miller W, Myers EW, Lipman DJ. Basic Local Alignement Search Tool SF. J Mol Biol 1990; 215:403-10.
16. Marti-Renom, MA. Stuart, A. Fiser, R. Sánchez, F. Melo, A. Sali. Comparative protein structure modeling of genes and genomes. Ann. Rev Biophys Biomol Struct 2000; 29:291-325.
17. RD. Finn, J. Mistry, B. Schuster-Böckler, S. Griffiths-Jones, V. Hollich, T. Lassmann, et al. Pfam: clans, web tools and services. Nucleic Acid Research 2006; 34:D247-D251.
18. YbdK is a carboxylate-amine ligase with a γ-glutamyl:cysteine ligase activity: crystal structure and enzymatic assays, Proteins: Struct, Funct Genet 2004; 56:376-83.
19. Schwede T, Kopp J, Guex N, Peitsch MC. "SWISS-MODEL: an automated protein homology-modelling server".Nucleic Acids Research 2003; 31:3381-5.
20. R. Guerois, JE. Nielsen and L. Serrano. Predicting changes in the stability of proteins and protein complexes: A study of more than 1000 mutations. J Mol Biol 2002; 320:369-87.