A Single Amino Acid Substitution (157 Gly → Val) in a Phosphoglycerate Kinase Variant (PGK Shizuoka) Associated With Chronic Hemolysis and Myoglobinuria

By Hisaichi Fujii, Hitoshi Kanno, Akira Hirono, Tadahiko Shiomura, and Shiro Miwa

We have determined a single amino acid substitution in a new phosphoglycerate kinase (PGK) variant, PGK Shizuoka, associated with chronic hemolysis and myoglobinuria. PGK Shizuoka had an extremely low enzyme activity with normal kinetic properties and normal electrophoretic mobility. Total blood cell RNA of the patient was reverse-transcribed and amplified by the polymerase chain reaction. A single nucleotide substitution from guanine to thymine at position 473 of the PGK messenger RNA was found. This nucleotide change causes a single amino acid substitution from Gly to Val at the 157th position, which is located in the NH₂-terminal domain of the enzyme. This mutation creates a new Bst XI cleavage site in exon 5, and we thus confirmed the mutation in the variant gene. The replacement of Gly by Val is considered to affect enzyme catalysis.

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PHOSPHOGLYCERATE kinase (PGK) is a key enzyme for ATP generation in the glycolytic pathway, and catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate. The complete amino acid sequence and nucleotide sequence of both cDNA and genomic DNA have already been elucidated. An inherited PGK deficiency is associated with nonspherocytic hemolytic anemia and also often with mental disorders. Cases from 14 unrelated families have been reported. The molecular defects of PGK variants associated with the enzyme deficiency have been identified in PGK München, PGK Uppsala, PGK Tokyo, and PGK Matsue. The correlation between the functional and structural abnormalities of these variants has been discussed. Recently, we discovered an additional case of PGK deficiency associated with chronic hemolysis and myoglobinuria. We report here the clinical, enzymatic, and molecular abnormality of the new variant, PGK Shizuoka. This is the first case of a PGK deficiency associated with both hemolysis and muscle symptoms.

MATERIALS AND METHODS

Case report. The patient, T.S., is a 27-year-old Japanese male. Nausea, anorexia, and muscle weakness after exercise had been present since the age of 10. At age 15 he had been admitted to the Shizuoka Central Hospital due to muscle weakness, vomiting, and jaundice after swimming. The diagnosis of exercise-induced myoglobinuria was made. At the age of 27 he had a similar episode and visited the Shizuoka General Hospital. His sclerae were slightly icteric. No sign of mental or growth retardation was observed.

Materials. All the substrates and nucleotides used for the determination of RBC enzyme activities, glycolytic intermediates, and adenine nucleotides were purchased from Boehringer-Mannheim (Mannheim, Germany). Dyematrex Gel Red A was a product of Amicon (Beverly, MA). Avian myeloblastosis virus reverse transcriptase was obtained from Seigakaku Kogyo (Tokyo, Japan). Restriction endonucleases and other modifying enzymes were from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Beverly, MA). Thermus aquaticus (Taq) polymerase was purchased from Cetus (Norwalk, CT). For sequencing, modifying T7 DNA polymerase (Sequenase; US Biochemicals, Cleveland, OH) was used.

Enzymatic analysis. RBC enzyme activities were determined by the method of the International Committee for Standardization in Haematology (ICSH). Reducit glutathione (GSH) was determined according to the method of ICSH. RBC glycolytic intermediates and adenine nucleotides were quantified enzymatically according to Minakami et al., except for 2,3-diphosphoglycerate and 1,3-diphosphoglycerate, which were assayed by the method of ICSH and Nakashima et al., respectively. The enzyme was partially purified by affinity chromatography with Dyematrex Gel Red A and concentrated by ultrafiltration. Kinetic and electrophoretic studies were performed as previously described. Oligonucleotides. Oligonucleotides were synthesized on a DNA Synthesizer (model 381A; Applied Biosystems, Foster City, CA) according to the published cDNA and genomic sequences. Sequences of the oligonucleotides used in this study are as follows: PGK-A, 5′-ACTAGTGAATCTTTGACATG-3′; PGK-B1, 5′-TTCGCGAAATCCCGACTCTC-3′; PGK-B2, 5′-CAGCATATTATGTAGGCTG-3′; PGK-C1, 5′-GCGGAGCTAAAGTTTGACAGACA-3′; PGK-C2, 5′-GAGTAAAAATCGTAAAGTTGAC-3′; PGK-D1, 5′-GAGGACTCTTGTGGTATGAATGCT-3′; PGK-D2, 5′-AATAGGAATACTGTTCACGTTG-3′; PGK-D3, 5′-AACACTTTGTGACAACTACT-3′. The polymerase chain reaction (PCR) was performed with the use of PGK-C1 and PGK-D1 primers. Pertinent laboratory data included the following: packed cell volume, 34.1%; red blood cell (RBC) count, 3.59 x 10⁶/mm³; hemoglobin (Hb) concentration, 12.8 g/100 mL; mean corpuscular volume, 95 µm³; reticulocyte count, 2.5%; serum total bilirubin, 2.7 mg/100 mL, of which 2.1 mg/100 mL was indirect reacting; bone marrow aspirate, marked erythroid hyperplasia. There was no family history of anemia or neuromuscular diseases and no known consanguinity in the family. Both parents and brother were normal.

From the Department of Blood Transfusion Medicine, Tokyo Women's Medical College; Okinaka Memorial Institute for Medical Research, Tokyo, Japan; and the Department of Hematology, Shizuoka General Hospital, Shizuoka, Japan.


Address reprint requests to Hisaichi Fujii, MD, Department of Blood Transfusion Medicine, Tokyo Women's Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162, Japan.

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PGK-C2 was digested with MboI and PstI. These cDNA fragments were then subcloned into a plasmid vector, pBluescript (Stratagene, La Jolla, CA), and sequenced by dideoxy chain termination. Analysis of restriction fragments of genomic DNA. Genomic DNAs were isolated from the patient, his parents, brother, and a normal control. The DNA fragments containing the mutation site were amplified from 0.5 μg of genomic DNA using a pair of primers corresponding to a 3' part of intron 4 (PGK-D1) and a 5' part of intron 5 (PGK-D2). The amplified DNAs were digested with BstXI according to the supplier's conditions. The digested and undigested DNA fragments were separated by 12% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining.

Results

Enzymatic analysis. Determination of RBC enzyme activities showed that the patient's RBC PGK activity was only 0.7% of the normal mean, being 2 IU/g Hb (normal range, 255 to 325). Other enzyme activities were either high or normal, reflecting the young mean RBC age. PGK activity of the mother was slightly low (231 IU/g Hb), while the father and brother had normal enzyme activity. The results of the determination of RBC glycolytic intermediates, adenine nucleotides, and GSH are shown in Fig 1. The accumulation of glycolytic intermediates above the PGK step was seen in the patient's RBCs. Dihydroxyacetone phosphate (DHAP) levels were increased 11-fold. The concentration of ATP was within normal range, but his Fru3P ATP level was considered low when compared with a comparable reticulocyte-rich control. The moderate increase in glyceraldehyde 3-phosphate, DHAP, and fructose 1,6-diphosphate was seen in the mother's RBCs.

Kinetic and electrophoretic studies were performed on a partially purified sample. The Michaelis constants for ATP of the normal and the patient's enzymes were 410 pmol/L and 330 pmol/L, respectively. Those for 3-phosphoglycerate of the normal and the patient's enzymes were 360 μmol/L and 385 μmol/L. The substrate affinity of the patient's enzyme was considered to be normal. The pH optimum of the patient's enzyme was similar to normal, both being pH 7.0. The thermal stability test at 45°C indicated that the patient's enzyme was as stable as that of the normal (data not shown). Electrophoresis of the patient's PGK showed the same mobility as that of the normal enzyme.

Determination of the molecular abnormality. Sequence analysis of the patient's cDNA showed a single nucleotide substitution from guanine to thymine at position 473 counting from the adenine at the initiation codon of PGK messenger RNA (mRNA). This nucleotide change causes a single amino acid substitution from Gly (GGG) to Val (GEG) at the 157th position from the NH2-terminal Ser residue, and creates a new Bst XI cleavage site in exon 5. The 229-bp amplified fragments containing the exon 5 sequence from the genomic DNA of the patient were digested completely into two fragments, 101 and 128 bp, by Bst XI. Half of the PCR products from the mother were digested, while those from his father, brother, and a normal subject were not cleaved (Fig 2).

Discussion

A hereditary PGK deficiency is associated with hemolytic anemia and often with neurologic manifestations. At the present time, 14 variants have been reported. Six of these patients have manifested both symptoms; four have shown only hemolysis; three have shown exercise-induced myoglobinuria but without hemolysis; and one case was without clinical symptoms. We elucidated single amino acid substitutions in PGK München, PGK Uppsala, and PGK Tokyo by micropeptide mapping and clarified the correlation between the functional and structural abnormalities of these variants (Fig 3). The substitutions that induced a diisocination of the nucleotide binding site of the enzyme

Fig 1. Results of the determination of RBC glycolytic intermediates, adenine nucleotides, and reduced glutathione of the patient (O-O) and his mother (O- -O). \( ^{1} \) Normal range. The following abbreviations are used: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F-1,6-DP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde 3-phosphate; 1,3-DPG, 1,3-diphosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; NAD, nicotinamide adenine dinucleotide; GSH, reduced glutathione.
resulted in markedly altered enzyme properties and severe clinical symptoms. Quite recently, a structural abnormality of the NH2-terminal domain, which is speculated to be the phosphoglycerate binding site, has been determined. This substitution was expected to induce structural instability and functional retardation.

The present variant, PGK Shizuoka, is associated with an RBC enzyme deficiency (less than 1% of normal activity), chronic hemolysis, and myoglobinuria. From the enzymatic studies, the normal and the patient’s enzymes were not significantly different with respect to their substrate affinity, pH optimum, thermal stability, and electrophoretic profile. The structural abnormality in PGK Shizuoka was a single nucleotide substitution from guanine to thymine at position

![Fig 3. Three-dimensional model of human PGK. Positions of the amino acid substitutions of reported variants and PGK Shizuoka are also shown.](image)
473. As shown in Fig 2, this mutation exists in the variant gene, and is carried by his mother. This nucleotide substitution predicts a Gly to Val at amino acid 157, which is located in a random coil of the catalytic domain (Fig 3). The replacement of the smallest amino acid, Gly, by the large Val residue seems to disturb the conformation of the enzyme and affect enzyme catalysis. It is, therefore, considered that PGK Shizuoka has extremely low activity despite the normal substrate affinity and normal stability.

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