Study of The Molecular Defects in Pyruvate Kinase Deficient Patients Affected by Nonspherocytic Hemolytic Anemia

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ABSTRACT. We have examined DNA from fifteen unrelated pyruvate kinase deficient patients with hereditary nonspherocytic hemolytic anemia (HNSHA) for the molecular alterations responsible for the enzyme deficiency. All but 3 of the 30 putative mutations were identified. Fourteen different mutations were found. Nine were missense mutations: 320 T→C, 823 G→C, 1276 C→T, 1376 C→T, 1378 G→A, 1484 C→T, 1529 G→A, 1654 G→A, 1675 C→G; three were nonsense mutations: 603 G→A, 721 G→T, 1501 C→T; one was an insertion at 1574 GGG→GGGG and the other a three nucleotide in-frame deletion 391-392-393 ATC. Eight of these mutations have not been previously described. We also investigated all of the patients for the C/A polymorphism at nt 1705 and the microsatellite ATT repeat in intron 11. All of the mutations that had previously been reported by us (391-393del, 721T, 1484T, 1529A) were found in the context of the same haplotype as the earlier cases, supporting the concept that each may have a single origin.

INTRODUCTION

Erythrocyte pyruvate kinase (PK) deficiency is the most common cause of hereditary nonspherocytic hemolytic anemia (HNSHA) (1). The clinical symptoms of the patients are variable, ranging from cases in which exchange transfusions are required, to those with a fully compensated hemolytic anemia. Splenectomy is often indicated in PK deficient patients with severe HNSHA; usually the response consists of an increase of levels of hemoglobin of 2-3 g/dL. In other cases the patients become transfusion-dependent and iron storage disease may occur (2).

Of the two pyruvate kinase genes present in humans, *PKLR* (coding for the L and R isoenzymes) (3) and *PKM2* (coding for the M1 and M2 isoenzymes) (4), only the former is normally expressed in erythrocytes and is therefore responsible for the deficiency in red cells. In this report we describe the molecular alterations of *PKLR* in a group of 15 unrelated patients with HNSHA who had low red cell PK activity.

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TABLE 1. Characteristics of Index Patients with Pyruvate Kinase Deficiency

#	Ethnic origin	PK activity (% of N)	Mutations	Amino Acid substitution	nt 1705	Micro-satellite
1	US black	25.9	1529G→A/**320T→C**	Arg510→Gln/Met107→Thr	C/C	10/14
2	Hispanic	45.9	1529G→A/ **1501C→T**	Arg510→Gln/Gln501→End	C/C	14/15
3	US white	20.5	**1654G→A**/721G→T	Val552→Met/Glu241→End	C/C	14/14
4	US white	52	1529G→A/1276 C→T	Arg510→Gln/Arg426→Trp	A/C	12/14
5	Hispanic	18.9	1378G→A/?	Val460→Met/?	A/C	12/16
6	unknown	11.8	1529G→A/721 G→T	Arg510→Gln/Gln241→End	C/C	14/14
7	Arab	94.4	**1675C→G/1675C→G**	Arg559→Gly/Arg559→Gly	C/C	15/15
8	Irish	15.1	1484C→T/**823G→C**	Ala495→Val/Gly275→Arg	C/C	15/17
9	Irish	21.4	391-393del/ **603G→A**	Ile131del/Trp 201→End	C/C	14/15
10	Irish	5.9	1529G→A/391-393del	Arg510→Gln/Ile131 del	C/C	14/15
11	US white	22.8	**1574G ins**/721G→T	Frameshift/Glu241→End	C/C	14/16
12	US white	20.6	1529G→A/1529G→A	Arg510→Gln/Arg510→Gln	C/C	14/14
13	US white	8.9	1529G→A/?	Arg510→Gln/?	C/C	14/14
14	US white	76.6	**1376C→T**/?	Ala459→Val/?	A/C	14/15
15	US white	16.1	1529G→A/1484C→T	Arg510→Gln/Ala495→Val	C/C	14/15

New mutations are shown in **bold** type. ? indicates that no mutation was found.

MATERIAL AND METHODS

Patients

Patients 6 and 11 were diagnosed at birth, when exchange transfusion was required. In several cases splenectomy had already been performed (8, 9 and 10) with apparent improvement. Other patients were being transfused regularly to permit them to reach an age at which the risks of splenectomy were acceptable.

The ethnic origin of the patients is presented in table 1. All the patients were of European ancestry except for one family of Middle Eastern origin. In patients 8, 9 and 10 biochemical characterization of the residual enzyme characteristics has been reported (5).

The PK activities are reported as a percentage of normal in table 1. Patients 2 and 11 were transfused one and two months respectively before the blood was drawn, so that the activity measured does not represent the patient's red cells alone, but also that of transfused cells.

DNA preparation and study

Leukocytes were isolated from peripheral blood and genomic DNA was extracted using standard manual methods or the Applied Biosystems (Foster City, CA) DNA extractor. Since in our previous study (6) the 1456T, 1484T and in particular 1529A mutations seemed to be common in the European population, the patients were initially screened for these mutations using restriction endonuclease analysis (7).

Single-strand conformation polymorphism (SSCP) (8) analysis was used to locate other mutations rapidly. When a band shift was found in a particular exon by SSCP, direct sequence analysis was performed to identify the mutation. When no SSCP abnormality was noted, the coding regions were sequenced until the mutations had been identified. The mutations reported were confirmed by the use of restriction endonuclease analysis (table 2 and 3) or by sequence analysis of both strands.

TABLE 2. Confirmation of pyruvate kinase mutations by PCR and endonuclease digestions

Mutation	Exon	Sense Oligo	Anti-Sense Oligo	Size PCR	Enzyme	Size Normal Frag.	Size Mutant Frag.
320T→C	4	A	B	133	Bcl I -	91+42	133
391-393 del	5	C	D	232 normal 229 mutant	Mow I +	9+223	9 + 64 + 156
721G→T	7	E	F	411	“Afl III” +	411	17 + 394
823G→C	7	E	F	411	Nci I +	86-87 +324-325	37-38 + 86-87 + 287
1276C→T	10	G	H	181	Nci I -	56-57 + 124-125	181
1376C→T	10	G	H	181	Mwo I -	9+9+19+60+84	9+60+112
1378G→A	10	G	H	181	NsiI	181	18+163
1484C→T	11	I	J	326	Mwo I -	25+68+96+137	68+121+137
1501C→T	11	I	J	326	Sty I +	326	115+211
1529G→A	11	I	J	326	Sty I +	326	143 + 183
1574G→GG	11	I	J	326 normal 327 mutant	Bgl I -	189+137	327
1654G→A	12	K	L	206	Scrf I -	47+35+28+96	47+63+96
1675C→G	12	K	L	206	Ava II +	206	100+106

Quotes indicate that an artificial restriction site has been introduced by using a mismatched oligonucleotide.

TABLE 3. Oligonucleotides used in confirmation of mutations

Sense Oligonucleotides	Antisense Oligonucleotides
A = 5'-GTTGCCTCTCATGTTCTGGG-3' C = 5'-CGAGGTCCTGGCCACCTTCC-3' E = 5'-GAGGGACTGGTGACCCA**C**GT-3' G = 5'-CCTTCCATACCCCAGTGCCC-3' I = 5'-CTCGTTCACCACTTTCTTGC-3' K = 5'-TGTGAGCCACCACACCTGTC-3'	B = 5'-TGGGAGAAGTTGAGTCGCGC-3' D = 5'-GCGCCGCCTTTCCGGCCCTG-3' F = 5'-CCCCCGTGCCACCATGA**G**GC-3' H = **5**'-CAGCACTTGAAGGCAGC**ATG**CA-3' J = 5'-GAGGCAAGGCCCTTTGAGTG-3' L = 5'-GGAAGGGATGGGGTACAAGG-3'

The bold font in the oligonucleotides represents a mismatched nucleotide.

Sequence of the promotor region

The putative promotor region was sequenced by asymmetric PCR (9). The oligonucleotides were selected from the published sequence of Kanno et al (10). Oligonucleotides 5'-ccaccatattatcacagtga-3' and 5'-ggctcctagttttcaccctc-3' were used in the first amplification (93°C 30 s denaturation, 58°C 30 s annealing and 72°C 30 s extension). The oligo-nucleotides 5'-ctccagccccaccctaca-3' and 5'-ggatgatatgttctcctggat-3' were used for single-strand amplification and sequencing.

Determination of the polymorphic sites

Analysis of the two known polymorphic sites was performed on all the patients and on some of the family members. The microsatellite ATT repeat located in intron 11 of the *PKLR* gene (11) was evaluated as previously reported (7). The 1705 C/A polymorphic site (12) was examined using restriction endonuclease analysis (13).

RESULTS

All but three of the 30 putative mutations in 15 unrelated pyruvate kinase deficient patients were identified. Eight of these were novel mutations. There were five missense mutations (320C, 823C, 1376T, 1654A, 1675G), two nonsense mutations (1501T, 603A), and one single nucleotide insertion (1574 GGG→GGGG). Patients 7 and 12 were the only ones found to be homozygous for a mutation. Patient 7 was one of three siblings homozygous for the 1675G mutation. The parents and the children of this family were studied for this mutation and for the two linked polymorphisms, C/A at nt 1705 and the intron 11 microsatellite. Figure 1 shows the pedigree of this family.

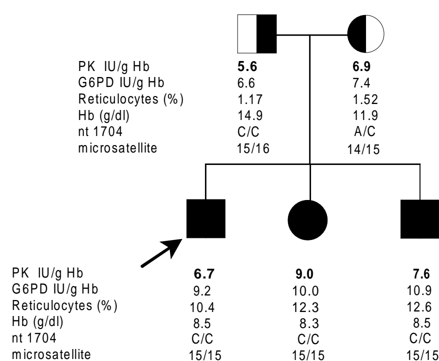

Figure 1. Findings in family members of patient #7 (arrow). A concurrent normal control had a G6PD activity of 7.5 IU/g Hb and a PK activity of 7.1 IU/g Hb. The high PK activity of the three children is the result of the decreased age of their circulating red cells.

Patient 13 was homozygous for the 1529A mutation, which was present in the heterozygous state in seven other patients, always with the same haplotype (1705C and 14 ATT repeat). The results of the haplotype analysis are reported in table 1.

Discrepancies with the published sequence

There were three discrepancies between the published sequence (10) of the putative promotor region and the sequence of the patients and a group of normal individuals. In each instance we found an insertion of one or two nucleotides, guanine between nucleotides 34 and 35 (10), adenine between nucleotide 41 and 42 and two adenines between nucleotide 55 and 56. At nt 265 we found a cytosine as has already been noted by Kanno et al (10) correcting the original sequence (14).

DISCUSSION

We identified 27 mutant genes with 14 different mutations by molecular analysis of 15 unrelated PK deficient patients. If all of the patients were homozygotes or mixed heterozygotes for PK deficiency, then 30 mutant alleles should exist. In three of the patients only one mutation was found. In one of these, patient 5, insufficient DNA was available to us to complete the sequence analysis, but in the case of patients 13 and 14 complete sequencing did not reveal the second mutation. It is possible that a mutation exists but was not found for technical reasons. It is also conceivable that in these patients the hemolytic anemia was not due to pyruvate kinase deficiency, but that these were patients that happened to be heterozygotes for PK deficiency who had hemolytic anemia for an unrelated reason. Clearly this will occur from time-to-time, since the gene frequency of pyruvate kinase deficiency is sufficiently high that it has been estimated that as many as 3% of some populations are heterozygous (15,16) although considerably lower frequencies have been reported in some other studies (17,18). In the case of patient 14 we had the opportunity to examine the blood of both parents. Each had approximately 60% of normal enzyme activity, a value that is well within the range found in heterozygotes.

Six of the mutations that were found have been reported previously (7,19); eight are new. Whenever a new mutation is identified it is important to ensure that it is the cause of the enzyme deficiency and not a polymorphic site or sporadic mutation without any phenotypic effect. The two nonsense mutations (603A, 1501T) and the single nucleotide insertion (1574 GGG→GGGG) are certainly the cause of the enzyme deficiency. The five mutations that cause

an amino acid substitution are initially only considered to be putative causes of the enzyme deficiency. Two of these amino acid changes are very drastic. The 823C mutation replaces the smallest amino acid (glycine) with a bulky positively-charged arginine. The reverse substitution occurs with 1675G mutation where arginine is replaced by glycine. In the remaining three mutations the changes are less substantial: the 320C mutation replaces a methionine with threonine, the 1376T mutation replaces an alanine with valine and the 1654A mutation a valine with methionine. In these cases we sequenced the entire coding region and the area of the putative promotor to ensure that no other mutations were present. Moreover when we compared these five mutated amino acids among several species all of them were well conserved (20).

The 823C mutation is the only one located near the putative active site (domain B, β-sheet 9 domain A, α-helix 3) determined by crystallographic studies of the cat muscle PK (20). It is interesting to note that the 1675G mutation (located in domain C of the protein, which seems to be responsible for the interaction between subunits) is the seventh mutation that causes a loss of an arginine in this area. This fact supports the hypothesis that arginine plays an important role in subunit contacts by creating salt bridges.

Of the 6 previously reported mutations, 5 had been encountered in patients studied earlier in our laboratory. The 1529A mutation, found nine times in this group of patients, was always in the same haplotype (microsatellite 14 ATT and nt 1705C). From this study and previous reports (7), it seems clear that in the European population one mutation (1529A) represents over 40% of the total, a small group of recurring mutations (391-392-393 ATT del, 721T, 1378A, 1456T, 1484T) represents about 4-6% and the rest are mutations that have been reported only once. The fact that each of the mutations found more than once is always in the same haplotype is compatible with the concept that each mutation has a single origin.

The frequency of the mutations in Japan is different from that in the European population; the 1529A mutation has never been encountered in a Japanese patient. Only very recently Kanno et al (19) reported a study of a group of Japanese patients in whom a 1468T mutation, which causes a loss of an arginine, was present in nine unrelated patients. So far the total number of mutations reported is 55 (6,7,12,14,19,21-31) and only three (1151T, 1276T and 1436A) of these have been found in both European and Asian populations. The pedigree of the family of patient #7 illustrates the potential value of pedigree analysis in the diagnosis of pyruvate kinase deficiency once the genotype of the parents and of one affected child was known. The nt 1704 C/A polymorphism would have been useful, since the propositus was homozygous for the C genotype and the mother was heterozygous. This would have excluded from having enzyme deficiency any children who had inherited the A allele from the mother, but there would still be a 50% probability that children who had the C/C genotype might be normal. However, the more diverse microsatellite locus provides unequivocal information in this family, because both parents were heterozygotes at this locus, viz. 15/16 and 14/15. Since the propositus was homozygous for the 15/15 genotype, all sibs and only those sibs with that genotype could be pyruvate kinase deficient.

Pyruvate kinase deficiency is one of the most difficult of the enzyme activities causing HNSHA to diagnose: in some patients in whom hemolysis is due to a mutant enzyme the catalytic activity of the enzyme in the red cells is not greatly reduced. Indeed, we and others have documented families in which HNSHA could be attributed to pyruvate kinase mutations, but in which the enzyme activity was actually elevated as measured in the laboratory (32). Such apparent anomalies probably occur for several reasons: 1) if the mutant enzyme is very unstable, the large number of young red cells in the blood can contribute sufficient enzyme activity to elevate the level well into the range usually associated with the heterozygous or even normal state; 2) kinetic abnormalities may render an enzyme ineffective *in vivo* even though it has high catalytic activity under the artificial conditions of laboratory assay; 3) the M isoenzyme may be expressed in the patient's red

cells (23,27) and this can contribute to the measured activity. The red cells of patient 7 had nearly normal PK activity as did both of his sibs. All three were homozygous for the 1675G mutation (figure 1). Patients 2 and 11 were transfused; the donors' erythrocytes were responsible, at least in part, for the relative high enzyme activity found in the patients. In patients such as these, molecular analysis may be very useful in confirming the diagnosis of PK deficiency.

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REFERENCES

1. Miwa S, Kanno H, Fujii H: Pyruvate kinase deficiency: Historical perspective and recent progress of molecular genetics. *Am J Hematol* 42:31-35, 1993.
2. Valentine WN, Tanaka KR, Paglia DE: Pyruvate kinase and other enzyme deficiency disorders of the erythrocyte. In: Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS, eds. *The Metabolic Basis of Inherited Disease*. New York: McGraw-Hill, Inc. p. 1606, 1983.
3. Noguchi T, Yamada K, Inoue H, Matsuda T, Tanaka T: The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. *J Biol Chem* 262:14366-14371, 1987.
4. Noguchi T, Inoue H, Tanaka T: The M_1- and M_2- type isoszymes of rare pyruvate kinase are produced from the same gene by alternative RNA splicing. *J Biol Chem* 261:13807-13812, 1986.
5. Elder GE, Lappin TRJ, Lawson BE, Bridges JM: Three pyruvate kinase variants with increased affinity for PEP. *Br J Haematol* 47:371-381, 1981.
6. Baronciani L, Beutler E: Analysis of pyruvate kinase-deficiency mutations that produce nonspherocytic hemolytic anemia. *Proc Natl Acad Sci USA* 90:4324-4327, 1993.
7. Baronciani L, Beutler E: Molecular study of pyruvate kinase deficient patients affected by nonspherocytic hemolytic anemia. *J Clin Invest* In press, 1995.
8. Orita M, Suzuki Y, Sekiya T, Hayashi K: Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5:874-879, 1989.
9. Beutler E, Kuhl W, Gelbart T, Forman L: DNA sequence abnormalities of human glucose-6-phosphate dehydrogenase variants. *J Biol Chem* 266:4145-4150, 1991.
10. Kanno H, Fujii H, Miwa S: Structural analysis of human pyruvate kinase L-gene and identification of the promoter activity in erythroid cells. *Biochem Biophys Res Commun* 188:516-523, 1992.
11. Lenzner C, Jacobasch G, Reis A, Thiele B, Nürnberg P: Trinucleotide repeat polymorphism at the PKLR locus. *Hum Mol Genet* 3:523, 1994.
12. Kanno H, Fujii H, Hirono A, Omine M, Miwa S: Identical point mutations of the R-type pyruvate kinase (PK) cDNA found in unrelated PK variants associated with hereditary hemolytic anemia. *Blood* 79:1347-1350, 1992.
13. Glenn D, Gelbart T, Beutler E: Tight linkage of pyruvate kinase (*PKLR*) and glucocerebrosidase (*GBA*) genes. *Hum Genet* 93:635-638, 1994.
14. Kanno H, Fujii H, Hirono A, Miwa S: cDNA cloning of human R-type pyruvate kinase and identification of a single amino acid substitution ($Thr^{384}\rightarrow$Met) affecting enzymatic stability in a pyruvate kinase variant (PK Tokyo) associated with hereditary hemolytic anemia. *Proc Natl Acad Sci USA* 88:8218-8221, 1991.
15. Abu-Melha AM, Ahmed MAM, Knox-Macaulay H, Al-Sowayan SA, El-Yahia A: Erythrocyte pyruvate kinase deficiency in newborns of Eastern Saudi Arabia. *Acta Haematol (Basel)* 85:192-194, 1991.
16. Feng CS, Tsang SS, Mak YT: Prevalence of pyruvate kinase deficiency among the Chinese: Determination by the quantitative assay. *Am J Hematol* 43:271-273, 1993.
17. Blume KG, Löhr GW, Praetsch O, Rüdiger HW: Beitrag zur Populationsgenetik der Pyruvatkinase menschlisher Erythrocyten. *Humangenetik* 6:261-265, 1968.
18. Mohrenweiser HW: Functional hemizygosity in the human genome: direct estimate from twelve erythrocyte enzyme loci. *Hum Genet* 77:241-245, 1987.
19. Kanno H: Molecular heterogeneity of pyruvate kinase deficiency identified by single strand conformational polymorphism (SSCP) analysis. *Blood* 84:13a, 1994.
20. Muirhead H, Clayden DA, Barford D, et al. The structure of cat muscle pyruvate kinase. *EMBO J* 5:475-481, 1986.
21. Neubauer B, Lakomek M, Winkler H, Parke M, Hofferbert S, Schröter W: Point mutations in the L-type pyruvate kinase gene of two children with hemolytic anemia caused by pyruvate kinase deficiency. *Blood* 77:1871-1875, 1991.
22. Kanno H, Fujii H, Miwa S: Low substrate affinity of pyruvate kinase variant (PK Sapporo) caused by a single amino acid substitution (426 Arg→Gln) associated with hereditary hemolytic anemia. *Blood* 81:2439-2441, 1993.

23. Kanno H, Fujii H, Tsujino G, Miwa S: Molecular basis of impaired pyruvate kinase isozyme conversion in erythroid cells: A single amino acid substitution near the active site and decreased mRNA content of the R-type PK. *Biochem Biophys Res Commun* 192:46-52, 1993.
24. Baronciani L, Beutler E: Analysis of pyruvate kinase (PK) mutations that produce nonspherocytic hemolytic anemia. *Blood* 82 (Suppl 1):97a, 1993.
25. Kanno H, Ballas SK, Miwa S, Fujii H, Bowman HS: Molecular abnormality of erythrocyte pyruvate kinase deficiency in the Amish. *Blood* 83:2311-2316, 1994.
26. Kanno H, Wei DCC, Miwa S, Chan LC, Fujii H: Identification of a 5'-splice site mutations and a missense mutation in homozygous pyruvate kinase deficiency cases found in Hong Kong. *Blood* 82 (Suppl 1):97a, 1993.
27. Lenzner C, Nürnberg P, Thiele B-J, et al. Mutations in the pyruvate kinase L gene in patients with hereditary hemolytic anemia. *Blood* 83:2817-2822, 1994.
28. Bianchi P, Terragna C, Zappa M, Alfinito F, Zanella A: Molecular characterization of *L-PK* gene in pyruvate kinase (PK) deficient Italian patients. *Blood* 84:14a, 1994.
29. Baronciani L, Westwood B, Beutler E: Study of the molecular defects in pyruvate kinase (PK) deficient patients affected by hereditary nonspherocytic hemolytic anemia (HNHA). *Clin Res (abstract) in press,* 1995.
30. Kanno H, Wei DC, Chan LC, et al. Hereditary hemolytic anemia caused by diverse point mutations of pyruvate kinase gene found in Japan and Hong Kong. *Blood* 84:3505-3509, 1994.
31. Pastore L, Della Morte R, Frisso G, Alfinito F, Rotoli B, Salvatore F: Alterazioni della forma R del gene della piruvato chinasi: presenza di una nuova mutazione (G1523) e bassa frequenza della A1529 in un campione dell'Itallia meridionale. *Analisi del DNA* 1995.
32. Beutler E, Forman L, Rios-Larrain E: Elevated pyruvate kinase activity in patients with hemolytic anemia due to red cell pyruvate kinase "deficiency". *Am J Med* 83:899-904, 1987.