

Studies on Erythrocyte Pyruvate Kinase (PK) Variant

1. Electrophoretic Screening for the Study of Genetic PK Polymorphism

Etsuko ODA

*Third Department of Internal Medicine,
Yamaguchi University School of
Medicine, Ube, 755, Japan*

(Received September 30, 1975)

INTRODUCTION

More than 135 cases of pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2. 7. 1. 40, PK) deficiency have been reported so far in international medical literature¹⁾. Recently, it has been discovered and later proven by kinetic studies that qualitative PK defect due to structural gene mutation causes hereditary hemolytic anemia^{2), 3), 4), 5)}. There have been a few reports on PK screening for quantitative deficiency^{6), 7)}, but not for qualitative defect since the kinetic study is not suitable for screening. Since electrophoresis is one of the valuable procedures for detecting the qualitative defect of mutant enzymes, variants of many erythrocyte enzymes such as glucose-6-phosphate dehydrogenase (G6PD) and phosphohexose isomerase (PHI) have been discovered by electrophoretic screening procedure. Further, the existence of electrophoretic variants with normal activity, such as G6PD A⁺^{8, 9)}, PHI 3-1, PHI 5-1 and PHI 6-1¹⁰⁾ were recognized. However, there have been no reports on PK screening by means of electrophoreses. Recently, a thin layer polyacrylamide gel electrophoresis for PK was developed by Imamura and Tanaka¹¹⁾ and it was proven to yield quite satisfactory results for identification of variant PKs^{12, 13)}. The electrophoresis is capable of revealing the existence of PK variant with different net electric charge, even when the mutant enzyme has normal activity, kinetics and stability.

In this paper, a thin layer polyacrylamide gel electrophoresis was applied to the screening of PK variants.

MATERIALS AND METHOD

Fresh donated blood specimens from 1,015 healthy adults anticoagulated with ACD (acid-citrate-dextrose) solution were supplied from

Yamaguchi Red Cross Center, Yamaguchi Prefecture, Japan. The whole blood was separated into platelet rich plasma and packed cells. The platelet rich plasma was used for platelet transfusion in our hospital ward and the unused packed red cells were used in this study. The packed cells were kept at 4°C and electrophoretic screening was performed within 72 hours after the harvest of blood. For separating leukocytes and platelets from erythrocytes, 2 ml of packed cells were washed with isotonic saline and centrifuged three times at 1,000×g for 5 min. Hemolysate was prepared according to the Nakashima et al.¹³⁾. The washed and packed erythrocytes thus obtained were suspended in an equal volume of 0.01% white saponin (E. Merck, Darmstadt, Germany) and mixed well. After centrifugation at 1,000×g for 10 min., the supernatant was added to a volume of toluene equal to one-fifth of the supernatant. The solution was shaken vigorously for 15 min. and was centrifuged again at 1,000×g for 15 min., and subsequently the toluene and stroma were removed. The method for electrophoresis with a thin layer polyacrylamide gel and the staining method for PK were according to those of Imamura and Tanaka¹¹⁾ with slight modifications as follows: A plate of polyacrylamide gel had 15 slots into each of which about 1 μl of sample was applied. The buffer consisted of 10 mM Tris-HCl (pH 8.2), 5 mM MgSO₄, 0.2 mM fructose 1, 6-diphosphate (FDP) and 50 mM mercaptoethanol and the reaction mixture contained 200 mM Tris-HCl (pH 7.4), 0.1 M KCl, 5 mM MgCl₂, 6 mM ethylenediaminetetraacetic acid (EDTA), 2.2 mM phosphoenolpyruvate (PEP), 5.5 mM ADP, 2 mM dihydronicotinamide-adenine dinucleotide (NADH), and 3.6 U/ml lactate dehydrogenase (LDH). Electrophoresis was carried out at 4°C, 27 V/cm for 5 hours.

When a sample with abnormal migration was found in the screening, more precise electrophoresis was performed. The hemolysate was prepared by the same way as the screening procedure except for the following modifications: Centrifugation was done at 4°C, 26,800×g for 15 min. by Hitachi Model 20-PR centrifuge after hemolysate was prepared using saponin. The hemolysate with toluence was shaken vigorously for 15 min., and then centrifuged at 4°C, 26,800×g for 15 min. Then the toluence and stroma were removed. The procedure for removal of stroma with toluence was repeated twice. The buffer contained 0.5 mM FDP and 0.5 mM dithiothreitol (DTT) instead of 0.2 mM FDP and 50 mM mercaptoethanol, respectively. A plate of polyacrylamide gel has 8 slots into each of which about 2 μl of sample was applied.

In conventional PK activity determination, the assay system employed 1.33 mM PEP, 2.0 mM ADP, 0.15 mM NADH, 8.0 mM MgSO₄,

6.0 mM EDTA, 75 mM KCl and 6.0 U/ml LDH in 50 mM triethanolamine-HCl buffer, pH 7.4 by the method of Bücher and Pfeleiderer¹⁴), with slight modifications. The assay was performed at 37°C, at 340 nm in a Hitachi Model 124 double beam spectrophotometer. Enzyme activity was expressed in units per 10^{10} red blood cells (RBC), where one unit was defined as the activity resulting in the conversion of one micromole of substrate per minute by 10^{10} RBC.

Routine hematological examinations were carried out by standard methods.

RESULTS

An electrophoretically abnormal erythrocyte PK pattern was observed in one out of the 1,015 healthy adults (0.099%) by erythrocyte electrophoretic screening (Fig. 1). The abnormal PK showed faster migration to the anode than the normal erythrocyte PK by both the conventional and the more precise electrophoresis as described in "Methods" (Fig. 2).

This specimen was obtained from H. F., a 35 year-old Japanese male. He has always been in good health, he was never known to have jaundice or anemia, nor was there any history of anemia or jaundice in his family. There is no evidence of consanguinity (Fig. 3).

Physical examination revealed a well developed male without the icteric sclera or hepatosplenomegaly.

The hematological and biochemical data at the time of the enzymatic studies are shown in Table 1. His hemoglobin was 15.7 g/dl, reticulocyte count was 1.6%, total serum bilirubin was 0.6 mg/dl and

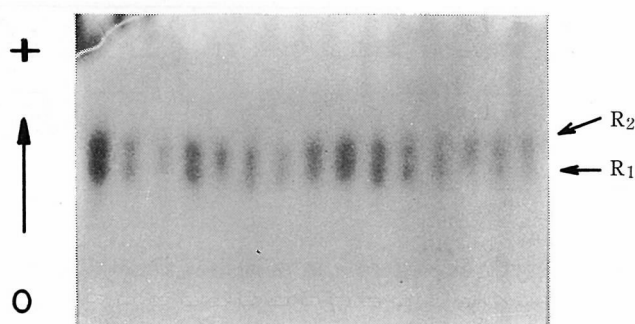


Fig. 1. Thin layer polyacrylamide gel electrophoresis of erythrocyte pyruvate kinase for screening. Supporting media: 3.34% acrylamide gel, 0.1 cm in thickness. Buffer: 10 mM Tris-HCl, 5 mM $MgSO_4$, 0.2mM FDP, 50mM mercaptoethanol, pH 8.2.

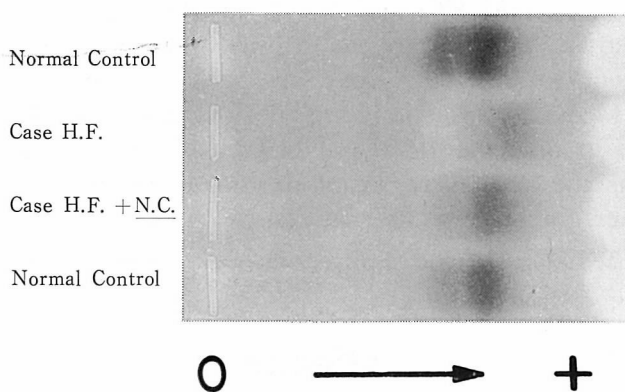


Fig. 2. Thin layer polyacrylamide gel electrophoresis of erythrocyte pyruvate kinase. H.F.: hemolysate of the propositus H.F. N.C.: normal control. Same procedure in Fig. 1 except for buffer: 10 mM Tris-HCl, 5 mM MgSO₄, 0.5 mM FDP, 50 mM mercaptoethanol.

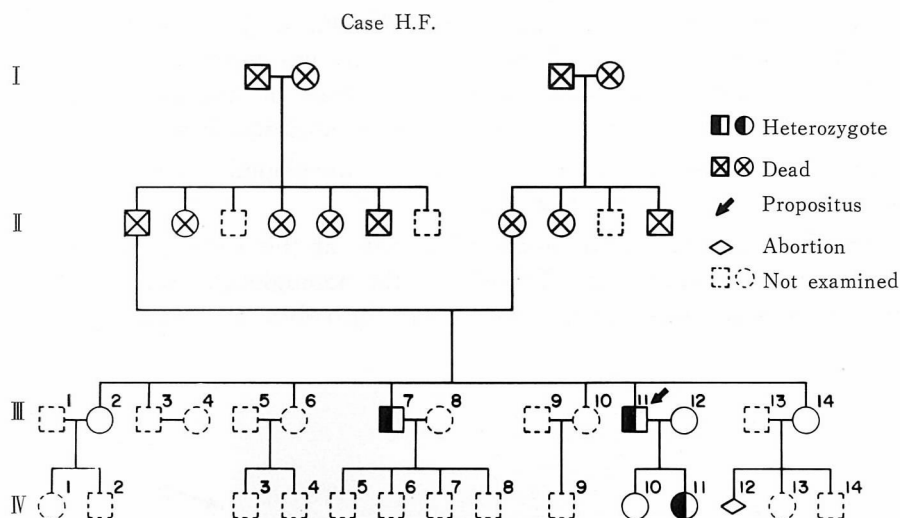


Fig. 3. Genealogical representation of propositus H.F.

the morphology of erythrocytes in the peripheral blood smear was normal, all of which reveal no evidence of hemolytic phenomena. There were no other significant abnormalities in the laboratory findings.

Erythrocyte PK activity of the propositus (H.F.) was 5.74 U/10¹⁰ RBC (normal range 3.34-6.42 U/10¹⁰ RBC).

Erythrocyte PKs of one of the propositus' elder brothers and one

Table 1. Hematological and biochemical data of propositus H.F.

HEMATOLOGICAL EXAMINATION			
RBC	$472 \times 10^4 / mm^3$	WBC	$4300 / mm^3$
Ht	45.6 %	N. band	2%
Hb	15.7 g/dl	N. seg	45.5%
MCH	33.2 $\mu\mu\text{g}$	Eosinophile	5%
MCV	97 μ^3	Basophile	0%
MCHC	34.4 %	Lymphocyte	40.5%
Retics	1.6 %	Monocyte	7%
Platelet	$27.3 \times 10^4 / mm^3$		
BLOOD CHEMISTRY TEST			
serum protein	7.6 g/dl		(6.5-8.0)
albumin-globulin ratio	1.53		(1.8-1.2)
icteric index	5		(4-6)
total bilirubin	0.6 mg/dl		(0.2-0.8)
cholinesterase	0.84		(0.75-1.1)
alkaline phosphatase	1.8u		(1-4)
cholesterol	253 mg/dl		(130-200)
GPT	22u		(5-33)
LDH	129u		(100-220)
leucine aminopeptidase	19u		(0-20)

of his daughters also showed faster migration as seen in the propositus by electrophoresis (Fig. 3). They also have been healthy. Erythrocyte PK activities of the elder brother and the daughter were 5.40 and 5.61 U/ 10^{10} RBC, respectively.

DISCUSSION

There have been a few reports as to the frequency of genetic quantitative abnormalities of pyruvate kinase. Until now the frequency had been estimated on the basis of erythrocyte PK activity. Blume et al.⁶⁾ found three heterozygotes among 214 Germans 1.4%. Helbig et al.⁷⁾ noted diminished erythrocyte PK activity in approximately 1% of 500 normal controls. Tanaka and Paglia¹⁾ found two of 146 normal individuals 1.37% in the heterozygous range of PK values.

However, there have been an increasing number of reports as to patients with qualitative PK defects and these reported patients did not always reveal low PK activities^{3, 5, 15)}. Also, there have been no reports on PK screening for qualitative defect.

Recently, the method of a thin layer polyacrylamide gel electrophor-

esis for PK was developed by Imamura and Tanaka¹¹. By this method, the electrophoretic abnormal mobility of erythrocyte PK in hemolytic anemia due to PK deficiency was clearly identifiable^{13, 16}.

Thus, in order to investigate the frequency of genetic variants of erythrocyte PK, electrophoretic screening was undertaken using thin layer polyacrylamide gel electrophoresis.

As shown in Fig. 2, a PK variant which had fast moving PK pattern in electrophoresis and normal erythrocyte PK activity was found in one of the 1,015 healthy blood donors. Although his parents had already died and were thus unavailable for study (Fig. 3), specimens from one of his elder brothers and one of his daughters revealed the same abnormal PK pattern as the propositus. These facts suggest that the propositus has the genetic PK defect in heterozygous form.

Among PK variants with abnormal electrophoretic patterns, until now, two heterozygotes with faster migration in electrophoresis have been reported^{13, 16}. These two cases were found during family studies of homozygous patients with hemolytic anemia. One is the father of PK Kiyose and the other is the mother of PK Sapporo. PK activities of the father of PK Kiyose and the mother of PK Sapporo were within normal range, 5.58 and 3.52 U/10¹⁰ RBC, respectively, and the mother of PK Sapporo revealed signs of mild hemolysis as proven by the presence of slight jaundice and reticulocytosis. The propositus in the present report and the father of PK Kiyose were asymptomatic. On examining electrophoresis and its activity, the erythrocyte PK of the propositus was found to be similar to that of the father of PK Kiyose. Hence, more precise examinations of erythrocyte PK of the propositus are necessary in order to clarify whether or not the PK variant in the propositus is identical to PK Kiyose. The results of further examinations will be described in second report.

The frequency of erythrocyte genetic PK defect was less when determined by electrophoretic mobility than by means of measuring the PK activity, the latter of which has been done in other studies^{1, 6, 7}. Among erythrocyte enzyme variants, there are cases which have normal electrophoretic patterns with low activity such as PK Maebashi¹⁷, as well as cases which have abnormal electrophoretic pattern with normal activity. While, it is possible that the prevalence discovered by means of measuring PK activity including acquired PK deficiency^{1, 18}, moreover, erythrocyte PK variants of heterozygotes do not always reveal the abnormal electrophoretic pattern as that of homozygotes. Hence, the difference between the frequency of erythrocyte genetic PK defect based

on PK activity determination and that based on electrophoretic mobility may be recognized. It is probably more reasonable to perform both electrophoresis and assay of PK activity in order to determine the frequency of PK genetic defect.

If a more advanced method of screening is developed, it is quite possible that the frequency of PK variants might be found to be higher.

SUMMARY

Erythrocyte pyruvate kinase screening study was carried out by means of a thin layer polyacrylamide gel electrophoresis. A new pyruvate kinase variant was found in one out of the 1,015 healthy Japanese adults examined in this study. The propositus was a 35 year-old, healthy male who showed no signs of hemolysis. Pyruvate kinase of the propositus migrated faster to anode than those of normal subjects, although the erythrocyte pyruvate kinase activity was revealed to be within normal range. The erythrocyte pyruvate kinase of one of his elder brothers and one of his daughters showed the same electrophoretic pattern as that of the propositus. The propositus was considered to be heterozygous for the new pyruvate kinase variant.

ACKNOWLEDGEMENT

I wish to thank Professor S. Miwa and Instructor K. Nakashima for their valuable guidance in this study and Mr. Titus S. Toyama for his review of English.

This study was supported in part by scientific research grants from the Ministry of Education (Nos. 837010, 857049).

A part of this report has been published in British Journal of Haematology 1975, 29; 157 by Miwa et al.

REFERENCES

- 1) Tanaka, K.R. and Paglia, D.E.: Pyruvate kinase deficiency. *Semin. Hematol.*, 8: 367-396, 1971.
- 2) Paglia, D.E., Valentine, W. N., Baughan, M.A., Miller, D.R., Reed, C.F. and McIntyre, O.R.: An inherited molecular lesion of erythrocyte pyruvate kinase. Identification of a kinetically aberrant isozyme associated with premature hemolysis. *J. Clin. Invest.*, 47: 1929-1946, 1968.
- 3) Paglia, D.E., Valentine, W.N. and Rucknagel, D.L.: Defective erythrocyte pyruvate kinase with impaired kinetics and reduced optimal activity. *Brit. J. Haematol.*, 22: 651-665, 1972.
- 4) Ohyama, H., Kumatori, T., Nishina, T. and Miwa, S.: Functionally abnormal pyruvate kinase in congenital hemolytic anemia. *Acta Haem. Jap.*, 32: 330-335, 1969.

- 5) Staal, G.E.J., Koster, J.F. and Nijessen, J.G.: A new variant of red blood cell pyruvate kinase deficiency. *Biochim. Biophys. Acta.*, 258 : 685-687, 1972.
- 6) Blume, K.G., Löhr, G.W., Praetsch, O., Rüdiger, H.W. and Wendt, G.G.: Beitrag zur Populationsgenetik der Pyruvatkinase menschlicher Erythrocyten. *Humangenetik.*, 62 : 261-265, 1968.
- 7) Helbig, W. and Jacobasch, G.: Sippenuntersuchung bei Pyruvatkinasemangelanämie. *Folia Haemat.*, 91 : 65, 1969.
- 8) Boyer, S.H., Porter, I.H. and Weilbacher, R.G.: Electrophoretic heterogeneity of G6PD and its relationship to enzyme deficiency in man. *Proc. Nat. Acad. Sci.*, 48 : 1868-1876, 1962.
- 9) Yoshida, A.: Human glucose-6-phosphate dehydrogenase: purification and characterization of Negro type variant (A⁺) and comparison with normal (B⁺). *Bioch. Genet.*, 1 : 81-99, 1967.
- 10) Detter, J.C., Ways, P.O., Giblett, E.R., Baughan, M.A., Hopkinson, D.A., Povey, S. and Harris, H.: Inherited variations in human phosphohexose isomerase. *Ann. Hum. Genet.*, 31 : 329-338, 1968.
- 11) Imamura, K. and Tanaka, T.: Multimolecular forms of pyruvate kinase from rat and other mammalian tissues. *J. Biochem.*, 71 : 1043-1051, 1972
- 12) Imamura, K., Tanaka, T., Nishina, T., Nakashima, K. and Miwa, S.: Studies on pyruvate kinase (PK) deficiency. II. Electrophoretic, kinetic, and immunological studies on pyruvate kinase of erythrocytes and other tissues. *J. Biochem.*, 74 : 1165-1175, 1973.
- 13) Nakashima, K., Miwa, S., Oda, S., Tanaka, T., Imamura, K. and Nishina, T.: Electrophoretic and kinetic studies on mutant erythrocyte pyruvate kinases. *Blood*, 43 : 537-548, 1974
- 14) Bucher, T. and Pfeleiderer, G.: Pyruvate kinase from muscle, Colowick, S.P. and Kaplan, N.O. (Eds.) : *Methods in Enzymology*, Vol. I., Academic Press, New York, pp. 435, 1955.
- 15) Miwa, S. and Nishina, T.: Studies on pyruvate kinase (PK) deficiency. I. Clinical, hematological and erythrocyte enzyme studies. *Acta Haem. Jap.*, 37 : 1-16, 1974.
- 16) Miwa, S.: Hereditary hemolytic anemia due to erythrocyte enzyme deficiency. *Acta Haem. Jap.*, 36 : 573-615, 1973.
- 17) Miwa, S., Nakashima, K., Ariyoshi, K., Shinohara, K., Oda, E. and Tanaka, T.: Four new pyruvate kinase (PK) variants and a classical PK deficiency. *Brit. J. Haematol.*, 29 : 157-169, 1975.
- 18) Arnold, H., Blume, K.G., Lohr, G.W., Boulard M. and Najean, Y.: "Acquired" red cell enzyme defects in hematological diseases. *Clin. Chim. Acta*, 57 : 187-189, 1974.