Severe hemolytic anemia in a Vietnamese family, associated with novel mutations in the gene encoding for pyruvate kinase

Catherine Costa
Juliette Albuisson
Le Thi Hao
Isabelle Max-Audit
Dinh Kiet Truong
Mario Tosi
Michel Goossens
Serge Pissard

Background and Objectives. Chronic hemolytic anemias are very frequent diseases in intertropical countries mainly caused by hemoglobin disorders. We studied a Vietnamese family in which a first child suffered from a severe transfusion-dependent anemia. The family requested an antenatal diagnosis during a second pregnancy. To characterize the molecular defect, we studied the family over three generations.

Design and Methods. Blood from family members was sampled for a full hematologic evaluation, including enzymatic dosage, and DNA analysis was performed for patients displaying pyruvate kinase deficiency (PK-R). Mutation research on the 11 exons of the *PKLR* gene was done using a scanning method and sequencing. Deletion was evidenced by a Sybergreen® based quantitative real time polymerase chain reaction (PCR) and mapped using quantitative multiplex PCR of short fluorescent fragments spread along the whole sequence of the *PKLR* gene.

Results. Hematologic and molecular studies of this severe chronic anemia demonstrated the existence of two defects in the *PKLR* gene, a new mutation located on exon 7: c.948C→G (N316K) and a large deletion extending from exon 4 to exon 10.

Interpretation and Conclusions. We describe a family in a South-east Asian country; the proband had severe transfusion-dependent chronic anemia caused by the association between two *PKLR* gene mutations, *PK Saigon* (N316K) and *PK Viet del 4-10*. Severe chronic anemia could be induced by various molecular defects mainly affecting the globin genes. However, even in populations in which hemoglobin diseases are frequent, enzymatic diseases should be considered.

Key words: *PKLR* gene, PK-R deficiency, quantitative PCR, QMPSF, Vietnam.

From the Laboratoire de Biochimie Génétique, AP-HP, et INSERM U468, Hôpital Henri Mondor, 94010 Créteil, France (CC, JA, IM-A, MG, SP); Department of Human Genetics, University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam (DKT, LTH); INSERM U614, Faculté de Médecine et de Pharmacie, Université de Rouen, France (MT).

*Correspondence:
Dr. Serge Pissard, Laboratoire de Biochimie Génétique, AP-HP, et INSERM U468, Hôpital Henri Mondor 94010 Créteil, France.
E-mail: serge.pissard@im3.inserm.fr*

Inherited chronic hemolytic anemia is the most frequent genetic disease worldwide and is mainly caused by defects in the α or β globin genes. However, other etiologies should be considered such as membrane defects and red blood cell (RBC) energy pathway deficiencies. Among this latter group of diseases, pyruvate kinase (PK-R) deficiency (EC: 2.7.1.40 and MIM: 266200) is caused by point mutations on the *PKLR* gene (chromosome 1q21). Since the first clinical description of PK-R deficiency,[1,2] more than 130 molecular variants have been described.[3] These variants are almost all rare or private mutations with only 3 mutations being found frequently: R486W, R510Q (ex 11) and E241X (ex 7). PK-R deficiencies lead to a wide range of disease severity depending on the effect of the mutation on the biological properties of the enzyme (allosteric regulation, tetramer of identical subunits). However, the relationship between the molecular defect and the enzymatic deficiency is not always understood.[4] Usually, carriers have a fully compensated mild chronic anemia. Homozygous or compound heterozygous patients have classical features ranging from mild anemia to a severe transfusion-dependent disease. Some cases of α-thalassemia-like disease with fetal loss due to *hydrops fetalis* have been described as being associated with PK-R deficiency.[5,6]

Design and Methods

Case report

A Vietnamese family, in which a first child (a 3-year old girl) was suffering from a severe chronic transfusion-dependent

Table 1. Standard hematologic data for the family.

	Hb (g/dL)	MCV fL	MCH	Hb F %	Hb A2 %	PKR value (UI/g Hb) nal: 5.9-8.1
Ia	13.1	64.4	22.5	1.9	6.8	na
Ib	15.2	88.1	31.6	< 0.2	4	na
IIa	13.2	83	30.5	< 0.2	3.1	3.6
IIb	14.3	91.2	34	< 0.2	3.4	4.3
IIIa*	na	na	na	na	na	na

Proband is regularly transfused since disease discovery and thus red cell indices cannot be interpreted in a reliable manner. Her transfusion history have caused a severe iron overload (ferritin 3424 mg /L (nl 12-80), transferrin saturation: 97% , sTFR: 57.4 nmol/L (8.7-28.1 nmol/L).

anemia, was investigated in order to discover the etiology of the disease during a subsequent pregnancy, as the family requested an antenatal diagnosis. The proband's disease was discovered at birth because of jaundice and the first transfusion was necessary at 3 days after a blood count showed severe anemia (4.5g Hb/dL and 17% hematocrit). The infant was then regularly transfused according to blood count. At present she has splenomegaly (7 cm) and hepatomegaly (3 cm) and a severe iron overload due to her transfusion history (ferritin 3424 mg/L, transferrin saturation: 97%). Apart from the proband, there was no history of severe anemia in this family. Three generations of the family were available for the study. Conventional blood tests were done on all members of the family. Blood samples for DNA extraction were taken only from patients with PK-R deficiency (proband, her parents and maternal grand-parents). Informed consent was obtained before sampling.

Red blood cell (RBC) phenotypic studies

RBC indices were measured by routine procedures. Studies for disease etiology included enzymatic activity assays performed on RBC purified from EDTA blood samples using cellulose columns as described by Beutler et al.[7] A PK-R deficiency was identified in both parents. Given her transfusion history, the proband's hematologic data cannot be interpreted. The biological data obtained in the family are presented in Table 1.

DNA sampling

DNA was extracted from peripheral blood leukocytes by standard procedures using the salting out method.

Mutation screening

Because of the high frequency of private mutations in PK-R deficiency,[3] the strategy used in our laboratory for detecting PKLR gene mutations is based on a

denaturing high performance liquid chromatography (D-HPLC) screening procedure of the 11 exons of the PKLR gene and 400 bp of the promoter using the Transgenomic's D-HPLC system (DNAsep® Cartridge ref DNA-99-3510 and Wave 2100®). Primers used in the study were designed so that the same polymerase chain reaction (PCR) conditions could be used for all fragments (PCR buffer: 50 mM KCl, 10 mM, tris HCl pH 7.9, 2 mM $MgCl_2$ and annealing at 60°C). Primer sequences and D-HPLC settings are available upon request. An abnormal pattern detected in exon 7 was identified using direct sequencing. Confirmation of the mutation was done by restriction fragment length polymorphism (RFLP) analysis since it creates a restriction site for the enzyme BsmaI (#R0529, N. E. Biolabs). Results of RFLP analysis were consistent with those of the D-HPLC study and DNA sequencing (data not shown). Mutations in the PKLR gene are designated using the numbering system designed by Bianchi and Zanella,[3] nucleotide numbering starts at position 40 of reference sequence GENBANK D13026 which is the A of the initiation codon.

Globin gene analysis

β-globin genes were analyzed using a routine PCR-DGGE strategy.[8] Deletional α-thalassemia was screened for using a multiplex-PCR method which detects 6 frequent α-thalassemic deletions (– $α^{3.7}$, –$α^{4.2}$, –$α^{20.5}$, – –MED, – –SEA, – –FIL).[9]

Filiation or chromosomal unisomy study

Microsatellites surrounding the PKLR gene location (1q21) were studied using the Applied Biosystems Linkage Mapping Set (ref 403089, Foster City, CA, USA) according to the manufacturer's instructions and run on an ABI 3100 capillary device. The results excluded a chromosome loss and familial inconsistency (Figure 1D).

Quantitative PCR

As D-HPLC analysis of the family led to the suspicion of a gene deletion removing exon 7 in the mother and child, we carried out real-time quantitative PCR to confirm this hypothesis. Exon 7 of the PKLR gene (1q21) and exon 1 of the β-globin gene (11p15, reference control) were amplified by real-time PCR in the proband, her parents and maternal grand parents, and 18 normal DNA controls. Relative and absolute quantifications were established by comparison with a range of serial dilutions of normal DNA (125, 25, 5, 1 ng/μL). PCR conditions were optimized ($MgCl_2$, primer concentration and annealing temperature) so that efficiency was similar. Primer sequences are as follows: PKLR forward: 5'GAGCAGGACGTCCGA-GACCT3', PKLR reverse: 5'GTGATGGGGAATAGC-
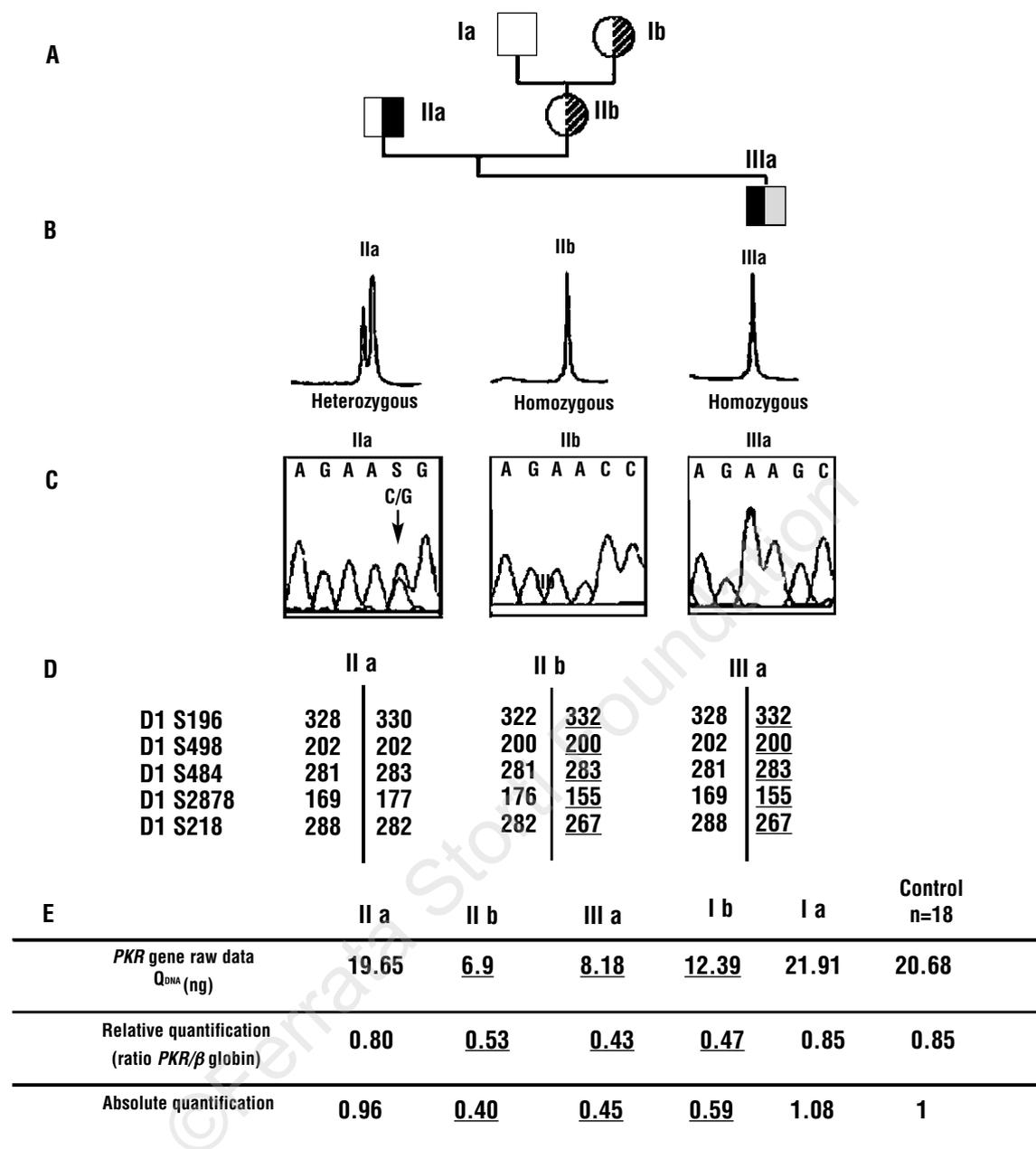
Figure 1. Molecular study of the family. Panel A. Family's pedigree. Patients IIa, IIb and IIIa are, respectively, the father, mother and proband. Panel B. D-HPLC pattern of exon 7. Primers and DHPLC settings for the 11 *PKLR* gene exons are available upon request. Panel C. Direct sequencing of exon 7. Father (IIa) is heterozygous for c.948 C→G (cDNA) N316K, whereas mother and child appear homozygous (or hemizygous) for c.948 C (wt) and nt 948 G (N316K), respectively. Panel D. Study of chromosome 1 microsatellites surrounding *PKLR* location (1q21) was performed to exclude parental unisomy and filiation inconsistency. Selected microsatellites are included in the ABI PRISM® Human linkage Mapping Sets from Applied Biosystems and used according to the kit user's manual. Panel E. Result of quantitative real-time PCR: the whole family, including maternal grandparents, was tested. Data show that the deletion is present in the maternal grandmother (Ia), the mother (IIb) and proband (IIIa) (underlined).

GACAG3', (220 bp) and for the β-globin gene forward: 5'TGCCAGAAGAGCCAAGGACA3'; β-globin reverse : 5'CTCACCACCAACTTCATCCAC3', (281 bp) (Genset, Paris, France). Reactions were carried out using a Light Cycler® instrument (Roche) with a reaction volume of 20 μL using the FastStart DNA Master SYBR Green I® Kit (Roche, Meylan, France), 3 mM $MgCl_2$, 0.5 μM of each primer, 50 ng of DNA from the patient or control and 125, 25, 5, and 1 ng of DNA calibrator. The PCR conditions were as follows: 8 min, 95°C, 95°C for 10s, 60°C for 10s, and 72°C for 15s (45 cycles). Two calibration curves were generat-
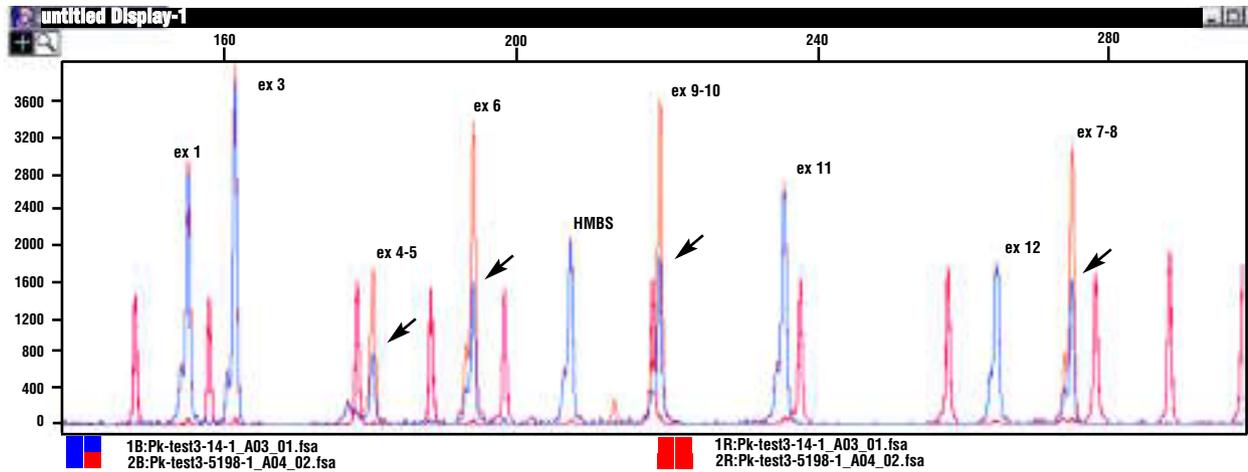
Figure 2. Detection of exonic deletions using QMPSF. The patient's chromatogram (blue line) is superimposed on the chromatogram of a normal control (orange line). The number above each peak indicates the exons covered by the fragment. Deleted exons show a height value roughly half the value of the normal control (indicated by an arrow head). The result shows a deletion covering from exon 4 to exon 10 The red line is the size ladder (Genescan™ 400HD [ROX], ABI, ref P/N 362246A).

ed for each gene with the DNA calibrators. These curves were used to quantify unknown samples. A *PKLR* gene/β-globin gene concentration ratio was calculated for relative quantification. A ratio between the patient's *PKLR* value and the mean control *PKLR* value was calculated for absolute quantification.

Results of the hematologic and molecular study of the *PKLR* gene in the family are presented in a single figure as a 5-panel flowchart (Figure 1).

Quantitative multiplex PCR of short fluorescent fragments (QMPSF)

In order to confirm the deletion of the exon 7 and to map the deletion, we set up a QMPSF assay as described by Charbonnier *et al*. and by Casilli *et al*.[10,11] A 9-fragment multiplex PCR, including one fragment not located on chromosome 1 (HMBS, located: 11q 23,3) used as a normalization control, was performed and run on an ABI 3100 capillary sequencer. The sequence of primers and the PCR conditions are available upon request. The patient's and normal control's chromatograms are superimposed and PCR efficiency adjusted with the normalization control fragment. Deleted regions show a peak height roughly half that of the control sample. The QMPSF experiment performed with the proband is shown in Figure 2.

Results

Hematologic data of the family showed a biological β-thalassemia trait only in the maternal grandmother. This common β⁰ thalassemia allele: codon 41-42 (TTCT) was not transmitted to the proband's mother and is thus not involved in the disease. Looking for

another etiology, we demonstrated the existence of a significant decrease in PK-R enzymatic activity compatible with a heterozygous PK-R deficiency in the mother and father (Table 1). Due to the ethnic origin of the family, we searched the proband's parents for a thalassemic trait. Apart from the grandmother's thalassemic trait, no β-thalassemic mutations or α-thalassemia deletions were found (*data available upon request*). These results are in agreement with the red cell indices of both parents.

During the molecular exploration of this family, we identified a new private mutation, c.948C→G (N316K) and, on the other allele, a deletion encompassing exon 7 of the *PKLR* gene. The c.948C→G mutation causes the change of a well conserved asparagine to lysine, introducing a positive charge next to the E315 (E271 in cat M1 pk) which is involved in the K⁺ and Mg⁺⁺ binding site and close to the catalytic site.[12-15] Thus, biochemical evidence correlates with family data as to the deleterious effect of this mutation. Evidence for the second molecular defect was found by chance, because it removes exon 7 producing a homozygous-like pattern in the mother and the affected child (Figures 1B and 1C). To confirm the existence of this large deletion, we set up a test using Sybergreen® dye and non-labeled primers in a real-time quantitative PCR. With this test, the relative quantity of the *PKLR* gene can be compared to that of a control (β-globin gene). As expected, the result showed a copy number of the *PKLR* gene about twice as low in the DNA of the patient (0.37), mother (0.46) and grandmother (0.51) as in that of the control (0.76), father (0.75) and grandfather (0.78). Absolute quantification of *PKLR* gene copies, evaluated by the ratio of patient's *PKLR*/mean *PKLR* of normal controls gave compara-

ble results, as it was about 2-fold lower in the proband's (0.45) mother's (0.40) and grandmother's DNA (0.59) than in control DNA (1.08), confirming the heterozygous status for the deletion. The results (Figure 1E), were confirmed using a QMPSF experiment showing the extent of the deletion which removes from exon 4 to 10 (Figure 2). Thus this deletion which removes 7 exons from the 11 exons of the *PKLR* gene is deleterious.

Discussion

Deletions are now recognized as common events in various pathologies, occurring in 1% to 30% of mutational events.[10,11,16-18] Analysis of the *PKLR* genomic DNA sequence with computer tools such as RepeatMasker (*http://ftp.genome.washington.edu/cgi-bin/RepeatMasker*) shows that around 50% of the sequence consist of various repeat sequences that may favor the occurrence of such mutational events. Most mutation detection methods, which are based upon the use of short PCR products, fail to detect such molecular lesions. To date, only one large deletion removing exon 11 has been described in PK-R deficiency.[19] Our molecular studies revealed that a new *PKLR* mutation located in exon 7 and a large deletion removing exon 7 were involved in this case. Confirmation of the deletion was obtained by a real-time quantitative PCR method using Sybergreen® dye instead of labeled probes, making this approach easily adaptable to any exon when a deletion is suspected. The real-time quantitative PCR method used in this investigation is fast, sensitive and automated, and thus a valuable alternative to the Southern blot technique. The use of real-time PCR with dedicated labeled probes has already been described for the detection of gene deletions in various diseases.[20,21] However, the strategy presented here does not require hybridization or hydrolysis probes and thus saves on the additional cost involved.[22] Futhermore, it is thought that the method could be rapidly adapted for almost any set of carefully designed primers, and thus extended to other genetic diseases. In intertropical countries, molecular defects in α or β globin genes causing thalassemia or Hb variant are very frequent etiologies of severe hereditary chronic anemias.[23] However red cell enzymatic deficiencies (G6PD or PK-R deficiency) are also frequent causes of such diseases[24,25] and can sometimes produce severe clinical manifestations. Therefore, enzymatic measurement should be included in etiologic investigations even in very severe or atypical diseases. Molecular studies to identify the anomalies involved in these pathologies are important: (i) in order to ascertain the diagnosis, which is sometimes doubtful with PK-R activity measurement and, therefore, to optimize the patient's care, and (ii) in cases of proven high severity, for genetic counseling and make an antenatal diagnosis available. PK-R deficiency is mainly caused by point mutations, most of which are private type and spread over the 11 exons of the gene. However, in this Vietnamese family, we demonstrated that the disease is caused by two new mutations, PK Saigon: c.948C→G (N316K) and a large deletion: del PK Viet (del 4 - 10). This finding highlights the need for methods suited specifically to detecting deletions and which are easy to use in a clinical laboratory setting. Finally, looking for the inheritance of the deletion in the family, we found that this large deletion was carried by the grand-mother who also carries a β⁰ thalassemia trait. Biochemical consequences of PK-R deficiency are decreased ATP or NADH availability in the red cells and an increase in the concentration of glycolytic intermediates such as 2,3 DPG. Such a situation has been found to cause the aggravation of other red cell pathologies such as sickle cell disease[26] and hereditary spherocytosis.[27]

However, as far as could be evaluated with the standard clinical and hematologic data available from this Vietnamese family, the association between a β⁰ thalassemia and the deleted *PKLR* allele did not lead to an aggravation of clinical symptoms in the grandmother.

References

1. Valentine WN, Tanaka KR, Miwa S. A specific erythrocyte glycolytic enzyme defect (pyruvate kinase) in three subjects with congenital non-spherocytic hemolytic anemia. Trans Assoc Am Physicians 1961;74:100-10.
2. Tanaka KR, Valentine WN, Miwa S. Pyruvate kinase (PK) deficiency hereditary nonspherocytic hemolytic anemia. Blood 1962;19:267-95
3. Bianchi P, Zanella A. Hematologically important mutations: red cell pyruvate kinase (third update). Blood Cells Mol Dis 2000;26:47-53.
4. Kahn A, Marie J, Vives-Corrons JL, Maigret P, Najman A. Search for a relationship between molecular anomalies of the mutant erythrocyte pyruvate kinase variants and their pathological expression. Hum Genet 1981;57:172-5.
5. Ferreira P, Morais L, Costa R, Resende C, Dias CP, Araujo F, et al. Hydrops fetalis associated with erythrocyte pyruvate kinase deficiency. Eur J Pediatr 2000; 159:481-2.
6. Gilsanz F, Vega MA, Gomez-Castillo E, Ruiz-Balda JA, Omenaca F. Fetal anaemia due to pyruvate kinase deficiency. Arch Dis Child 1993;69:523-4.
7. Beutler E, Blume KG, Kaplan JC, Lohr

GW, Ramot B, Valentine WN. International Committee for Standardization in Haematology: recommended methods for red-cell enzyme analysis. Br J Haematol 1977;35:331-40.

8. Ghanem N, Girodon E, Vidaud M, Martin J, Fanen P, Plassa F, et al. A comprehensive scanning method for rapid detection of β-globin gene mutations and polymorphisms. Hum Mutat 1992; 1:229-39.

9. Chong SS, Boehm CD, Higgs DR, Cutting GR. Single-tube multiplex-PCR screen for common deletional determinants of α-thalassemia. Blood 2000;95:360-2.

10. Charbonnier F, Olschwang S, Wang Q, Boisson C, Martin C, Buisine MP, et al. MSH2 in contrast to MLH1 and MSH6 is frequently inactivated by exonic and promoter rearrangements in hereditary nonpolyposis colorectal cancer. Cancer Res 2002;62:848-53.

11. Casilli F, Di Rocco ZC, Gad S, Tournier I, Stoppa-Lyonnet D, Frebourg T, et al. Rapid detection of novel BRCA1 rearrangements in high-risk breast-ovarian cancer families using multiplex PCR of short fluorescent fragments. Hum Mutat 2002; 20:218-26.

12. Muirhead H, Clayden DA, Barford D, Lorimer CG, Fothergill-Gilmore LA, Schiltz E, et al. The structure of cat muscle pyruvate kinase. Embo J 1986; 5:475-81.

13. Larsen TM, Laughlin LT, Holden HM, Rayment I, Reed GH. Structure of rabbit muscle pyruvate kinase complexed with Mn2+, K+, and pyruvate. Biochemistry 1994;33:6301-9.

14. van Solinge WW, Kraaijenhagen RJ, Rijksen G, van Wijk R, Stoffer BB, Gajhede M, et al. Molecular modelling of human red blood cell pyruvate kinase: structural implications of a novel G1091 to a mutation causing severe nonspherocytic hemolytic anemia. Blood 1997;90:4987-95.

15. Torshin IY. Functional maps of the junctions between interglobular contacts and active sites in glycolytic enzymes: a comparative analysis of the biochemical and structural data. Med Sci Monit 2002;8:BR123-35.

16. Audrezet MP, Chen JM, Raguenes O, Chuzhanova N, Giteau K, Le Marechal C, et al. Genomic rearrangements in the CFTR gene: extensive allelic heterogeneity and diverse mutational mechanisms. Hum Mutat 2004;23: 343-57.

17. Houdayer C, Gauthier-Villars M, Lauge A, Pages-Berhouet S, Dehainault C, Caux-Moncoutier V, et al. Comprehensive screening for constitutional RB1 mutations by DHPLC and QMPSF. Hum Mutat 2004;23:193-202.

18. Hofmann W, Gorgens H, John A, Horn D, Huttner C, Arnold N, et al. Screening for large rearrangements of the BRCA1 gene in German breast or ovarian cancer families using semi-quantitative multiplex PCR method. Hum Mutat 2003;22:103-4.

19. Baronciani L, Beutler E. Molecular study of pyruvate kinase deficient patients with hereditary nonspherocytic hemolytic anemia. J Clin Invest 1995;95:1702-9.

20. Costes B, Girodon E, Vidaud D, Flori E, Ardalan A, Conteville P, et al. Prenatal detection by real-time quantitative PCR and characterization of a new CFTR deletion, 3600+15kbdel5.3kb (or CFTR dele19). Clin Chem 2000;46:1417-20.

21. Hedrich K, Kann M, Lanthaler AJ, Dalski A, Eskelson C, Landt O, et al. The importance of gene dosage studies: mutational analysis of the parkin gene in early-onset parkinsonism. Hum Mol Genet 2001;10:1649-56.

22. Armour JA, Barton DE, Cockburn DJ, Taylor GR. The detection of large deletions or duplications in genomic DNA. Hum Mutat 2002;20:325-37.

23. Stamatyannopoulos G. The Thalassemia. In: G. S, Nienhuis AW, Majerus PW, Varmus H, editors. The Molecular Basis of Blood Disease. Vol. 1. Philadelphia: W.B. Saunders Co; 1994. p. 157-206.

24. Beutler E. The molecular biology of enzymes of erythrocyte metabolism. In: Nienhuis AW, Majerus PW, Varmus H, eds. The Molecular Basis of Blood Disease. Vol. 1. Philadelphia: W. B. Saunders Co; 1994. p. 331-50.

25. Miwa S, Fujii H. Pyruvate kinase deficiency. Clin Biochem 1990;23:155-7.

26. Cohen-Solal M, Prehu C, Wajcman H, Poyart C, Bardakdjian-Michau J, Kister J, et al. A new sickle cell disease phenotype associating Hb S trait, severe pyruvate kinase deficiency (PK Conakry), and an α2 globin gene variant (Hb Conakry). Br J Haematol 1998; 103:950-6.

27. Zarza R, Moscardo M, Alvarez R, Garcia J, Morey M, Pujades A, et al. Co-existence of hereditary spherocytosis and a new red cell pyruvate kinase variant: PK mallorca. Haematologica 2000;85:227-32.