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

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, more than 150 molecular variants have been described. 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. 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.

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 anemia, requested an antenatal diagnosis during their second pregnancy. To characterize the molecular defect, we studied the family over three generations.

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.

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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%), sTFR: 57.4 nmol/L (8.7-28.1 nmol/L).

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.’ A PK-R deficiency was identified in both parents. Given her transfusion history, the proband’s hematologic data 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%). 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.

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, 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 strategy used in our laboratory, the proband's hematologic data cannot be interpreted. The biological data obtained in the family are presented in Table 1.

**Table 1. Standard hematologic data for the family.**

<table>
<thead>
<tr>
<th></th>
<th>Hb (g/dL)</th>
<th>MCH</th>
<th>MCV</th>
<th>MCHC</th>
<th>Hb F</th>
<th>Hba2</th>
<th>PKLR value (UI/g Hb)</th>
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<td>64.4</td>
<td>22.5</td>
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<td></td>
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<tr>
<td>lb</td>
<td>15.2</td>
<td>88.1</td>
<td>31.6</td>
<td>&lt; 0.2</td>
<td>4.3</td>
<td>na</td>
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<tr>
<td>Ila</td>
<td>13.2</td>
<td>83</td>
<td>30.5</td>
<td>&lt; 0.2</td>
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<tr>
<td>Iib</td>
<td>14.3</td>
<td>91.2</td>
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<td>IIIa</td>
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*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, transferrin saturation: 97%)*

Filiation or chromosomal unisomy study

Microsatellites surrounding the PKLR gene location (1q21) and exon 1 of the β-globin gene (11p15, referred to as the TATA box) 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 (MgCl2, primer concentration and annealing temperature) so that efficiency was similar. Primer sequences are as follows: PKLR forward: 5’GAGCAGGAGCTCCGAGCACCT3’, PKLR reverse: 5’GTGATGGGGAATAGC-
GACAG3', (220 bp) and for the β-globin gene forward: 5'TGCCAGAAGAGCCAAGGACA3'; β-globin reverse: 5'CTCACCACAACTTCATCCCA3', (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 MgCl₂, 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-
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. 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 β-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 Mg2+ binding site and close to the catalytic site. 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 50% 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 Furthermore, 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 diseases24,25 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 β-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 disease26 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 β-thalassemia and the deleted PKLR allele did not lead to an aggravation of clinical symptoms in the grandmother.

References

7. Beutler E, Blume KG, Kaplan JC, Lohr CC: setup and performing sybergreen quantitative PCR; JA: student involved in the standard molecular study (DHPLC, RFLP, microsatellites); LTH: Vietnamese hematologist which managed family data and sampling; MA: enzymatic measurements; RFLP setting of QMPSF; SP: overall management, performing QMPSF test, writing the paper; DKT, MG: Vietnamese and French senior physicians. We are indebted to Dr. Henri Wacjman for helping us to improve our manuscript and to M. David Kerridge for his valuable assistance in editing the manuscript.

A substantial part of this work was used in a Poster presentation at the “European Society of Human Genetics” annual congress, May 2003, Birmingham: "PK deficiency: discovery of a new deletion” but there are no other publications of the full data. The authors declare that they have no potential conflicts of interest.


