Assessment of Genetics Mutations G1529A and G1168A in PK-LR gene in Neonatal Jaundice

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Abstract: Introduction: Pyruvate kinase (PK) deficiency is the most frequent red cell enzymatic defect responsible for hereditary non-spherocytic hemolytic anemia. It is one of the etiologic factors in newborns with jaundice. The aim of this study is to determine the prevalence of pyruvate kinase deficiency in Azeri population from North-West of Iran. Materials and Methods: In this descriptive study, 200 newborns with non-conjugated hyperbilirubinemia were included from a total of 1750 admitted newborns in 3rd referral university hospital. Routine Lab results were collected from hospital records. PK activity was determined by Coupled Enzyme Assay with using the ELISA technique. Results: The normal range of pyruvate kinase activity in 30 healthy samples of umbilical cord blood determined from 3.52-8.45 miliunit. In 32 out of 200 newborns (16%), there was more than 60% decrease in PK activity. Conclusion: This study shows a relatively high prevalence of PK deficiency in newborns with jaundice in north western Iran. Further molecular studies are recommended in this population.

Keywords: Neonatal Jaundice, Pyruvate Kinase Deficiency, G1529A, G1168A, PCR

1. Introduction

Elevated bilirubin in the newborns is a major problem in the world. The estimation of the prevalence of autosomal recessive diseases is a challenge controversial and there are so many of hyperbilirubinemia in newborns, most of which are hereditary aspects [1]. Except that X-linked G6PD deficiency and phosphoglycerate kinase, 12 red blood cell enzyme defects are that cause non-spherocytic hemolytic anemia [2]. 80% of hereditary non spherocyt hemolytic anemia due to lack of pyruvate kinase and G6PD deficiency caused [1, 3-5]. Pyruvate kinase (PK) deficiency is of interest because of its role in hereditary non-spherocytic hemolytic anemia [4-7]. This is inherited as an autosomal recessive disease, and clinical symptoms can be seen in patients with homozygous or compound heterozygote for two mutant alleles in PK-LR gene [6-10]. Phenotypically it exhibits a variable range of anemia, from mild to transfusion-dependent, in infants and children and tends to improve with age [11].

In these disease different symptoms such as anemia, reticulocytosis, hemolysis, elevated bilirubin, decreased haptoglobin, and increased lactate dehydrogenase has been reported [12-19]. Other symptoms include enlargement of spleen and bile gallstones [10]. PK is a key enzyme in Embden Meyerhoff pathway of glycolysis in humans with catalysis ATP production from ADP. Red cell PK catalysis the last step of the glycolytic pathway [20-25]. Its gene is located on chromosome 1q21 and contains 12 exons [26-28]. More than 180 different mutations in the PK-LR gene have been identified, which mostly consists of point mutations [29]. Its prevalence and mutations have been studied in different ethnic groups [2]. There are few studies have been done in Iran [9]. In this research, the frequency of PK deficiency was studied in newborns with jaundice in Azeri population from north western Iran.
2. Materials and Methods

2.1. Case Selection

In a five-month period from September 2014 to February 2015, 200 out of 1750 newborn admitted to our hospital, had unconjugated hyperbilirubinemia. Total bilirubin level required for enrollment was ≥17 mg/dl, necessitating further investigation and therapy. The total and direct bilirubin, hemoglobin concentration, hematocrit, MCV, red blood cell and reticulocytes count, blood group and Rh were determined by standard methods. The exclusion criteria’s were; newborns less than 35 weeks gestational age, the presence of cholestasis (direct hyperbilirubinemia), haemoglobinopathies, polycythemia, birth trauma, ABO, Rh blood group incompatibility and G6PD deficiency. None of the newborns had been transfused. After getting informed consent from parents, 3 ml of whole blood were collected in vials containing EDTA.

2.2. ELISA Assay

The activity of PK was measured by pyruvate kinase activity assay kit (Sigma Aldridge, USA, LOT number: B9C280709C) and was performed according to the kit manufacturer. In order to determine the normal mean and range of enzyme activity in newborns, 30 umbilical cord blood samples from healthy newborns without jaundice were collected.

For sample preparation, a 0.5 cc of blood samples from patients with hemolysis transferred to a test tube and were centrifuged at around 1000 rpm for 15 minutes and the plasma layer and buffy coat were removed. Samples of normal saline was centrifuged and washed three times with the same round. The operation to remove white blood cells that cause interference and false increase in the measurement of the activity of pyruvate kinase took place. According to protocol, measuring the activity of the enzyme pyruvate kinase to be in the number of red blood cells were done two million cells. Lubricating with 1 to 4 in a final volume of 50 ml, red blood cells were lysed.

Pyruvate kinase enzyme-substrate lyophilized Powder was mixed for each 220 ml with distilled water and was used. Positive control of pyruvate kinase in 100 ml of distilled water mixture was used. To produce 1 nmol obey ml, 10 ml of 100nmol/µL in 990 microliter buffer follow standard measure have diluted pyruvate kinase. The amounts of 8, 6, 4, 2 and 10 ml of standard solution 1nmol /µL added into the wells and pyruvate kinase buffer to a final volume of 50 ml was reached.
Table 1. Reagent necessary to measure the required quantities of enzyme activity PK.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Sample and Standards</th>
<th>Blank Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate kinase Assay Buffer</td>
<td>44µL</td>
<td>46µL</td>
</tr>
<tr>
<td>Pyruvate kinase Substrate Mix</td>
<td>2µL</td>
<td>-</td>
</tr>
<tr>
<td>Pyruvate kinase Enzyme Mix</td>
<td>2µL</td>
<td>2µL</td>
</tr>
<tr>
<td>Fluorescent Peroxidase Substrate</td>
<td>2µL</td>
<td>2µL</td>
</tr>
</tbody>
</table>

The data from the study, using descriptive statistics (frequency, percentage, mean and standard deviation), mean difference test for independent groups and test chi-square test or Fisher's exact test and logistic regression model using SPSS software IBM SPSS 21 were evaluated and statistical analysis. In this study, a statistically significant P-value less than 0.05 were considered.

Figure 3. EMP pathway.

3. Results

The mean normal for PK enzyme activity in 30 samples of healthy umbilical cord blood was 6.01 mU and range of 3.52-8.45 mU. Two hundred cases (115 boys and 85 girls) admitted with non-conjugated hyperbilirubinemia, enrolled in the study. 32 cases (16%) had ≥ 60% mean normal in PK activity and considered to be PK-deficient. The mean of total bilirubin, PK activity and hematocrit values in PK- deficient and PK-sufficient patients are shown in table 2.

Table 2. Laboratory parameters in PK sufficient and deficient newborns.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PK-sufficient patients (n= 168)</th>
<th>PK-deficient Patient (n=32)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Bilirubin (mg/dl)</td>
<td>19.5</td>
<td>21.8</td>
<td>0.374</td>
</tr>
<tr>
<td>Direct Bilirubin (mg/dl)</td>
<td>0.50</td>
<td>0.52</td>
<td>0.301</td>
</tr>
<tr>
<td>Direct Bilirubin (mg/dl)</td>
<td>18.95</td>
<td>21.28</td>
<td>0.370</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>17.6</td>
<td>17.9</td>
<td>0.467</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>48.6</td>
<td>51.1</td>
<td>0.376</td>
</tr>
<tr>
<td>Mean PK Activity (mU)</td>
<td>4.8</td>
<td>1.9</td>
<td>0.367</td>
</tr>
<tr>
<td>Range of PK activity</td>
<td>3.55-7.23</td>
<td>1.39-2.19</td>
<td></td>
</tr>
</tbody>
</table>

Hb: hemoglobin, HCT: Hematocrit, PK: Pyruvate Kinase
Using Pearson correlation coefficient and total bilirubin relationship PK enzyme activity was investigated. Contents Figure 1 shows that there is a negative correlation between the two variables (p>0.05)

Based on the results, despite showing a negative relationship, the relationship between two variables Total Bilirubin with PK activity is not observed in patients PK deficient. (p>0.05 r=-0.120*)

Figure 1. Relationship between the Total Bilirubin with PK activity.

Figure 2. Relationship between MCV with PK activity.
Based on the results graph the relationship between variables MCV with PK activity in the PK deficient patients is observed (p>0.05, r =-0.412*).

Figure 3. The relationship between MCH with PK activity.

Based on the above graph, the relationship between variable MCH with PK activity in PK deficient patients is observed (p>0.01 r =-0.558*).

Figure 4. The relationship between MCHC with PK activity.
Based on the above graph, the relationship between variable MCHC with PK activity in PK deficient patients is observed ($p>0.05$, $r=-0.358^*$).

In the case of quantitative variables with normal results showed that total bilirubin concentration, the activity of pyruvate kinase and hematocrit values between the two groups of PK-deficient and PK-sufficient patients there is a significant difference ($p>0.05$).

**Figure 5.** Shows that the values Pyruvate Kinase Activity between the two groups PK deficiency (mean: 1.98 mu/ml) and PK sufficient patients (Mean: 4.78 mu/ml) significant difference. ($P>0.05$).
Figure 6. Shows that total bilirubin levels between the two groups with the PK deficiency (mean: 21.82 mg/dl) and PK sufficient patients (Mean: 19.51 mg/dl) has significant difference. (P> 0.05).
Figure 7. Schematic representation of band formation pattern in G1529A genetic mutation compared to healthy genes.

Figure 8. Schematic representation of band formation pattern in G1168A genetic mutation compared to healthy genes.

4. Discussion and Conclusion

Many studies have been done on the causes and prevention jaundice with minimal damage to be necessary in order to fix the problem [29-41]. Now the phototherapy treatment methods commonly used to reduce bilirubin [42]. The estimation of the prevalence of autosomal recessive diseases are a challenge controversial and there are so many of hyperbilirubinemia in newborns, most of which are hereditary aspects such as hemoglobinopathies, enzymopathies and diseases of red blood cell membrane [13].

Disruption in the activity of the enzyme pyruvate kinase in human red blood cells are offered for different anomalies in a way that causes polycythemia and increased activity led to a deficiency of the enzyme pyruvate kinase deficiency hemolytic anemia is.

Pyruvate kinase deficiency is an autosomal recessive disorder. In this disease, anemia, reticulocytosis, hemolysis, elevated bilirubin, decreased haptoglobin, and increased lactate dehydrogenase has been reported [2, 43-45].

Our study showed that 16% of newborns with unconjugated hyperbilirubinemia are PK deficient in Azeri population of northwestern Iran. Pyruvate kinase activity in patients 1.39-2.19 Miliiunit /ml (mU), while PK sufficient patients activity of the enzyme in newborns 3.55-7.23 Miliiunit /ml respectively. Negative relationship between variables in patients with pyruvate kinase deficiency pyruvate kinase activity and total bilirubin were observed. 6 cases of patient's has been enzymatic activity less than 2 Miliiunit / ml. Variables MCV, MCH, Hct and MCHC, PK enzyme activity was inversely associated with hemolysis and hemolytic anemia indicating that PK is caused by a deficiency.

Figure 9. Schematic view of the frequency map of the prevalence of jaundice in the cities of Iran.

Figure 10. A circular diagram of the difference in the genetic mutations in the PK-LR gene in Asia (Japan) and Europe and the United States.
Similar study in non-Azeri population of southwestern Iran indicated that 22 out of 211 newborns (10.4%) had PK deficiency [9]. Similar results from China, India and Germany were 3.4, 3.2 and 1.4% respectively [12, 33]. In this study, the prevalence of G6PD deficiency was 2%, which is in line with the previous report in general Azeri population in northwest of Iran that was 2.28%.

5. Conclusion

the result of pyruvate kinase deficiency is the perfect addition to G6PD, pyruvate kinase deficiency also have to be a separate diagnosis of hemolytic anemia non spherocytic in neonates with jaundice also be considered in genetic counseling Premarital. In the end, it is suggested that the study on a wider level in infants with conjugated hyperbilirubinemia bread and the presence of the mutations in the genes for pyruvate kinase molecular studies need to be performed.

![Figure 11. Schematic View of Genetic Pathways in Pyruvic Acid Synthesis.](image)

References


