Abstract. – Objectives: Alpha-thalassemia (alpha-thal) is one of the most common genetic disorders and in some populations has prevalence as high as 30%. Disorders in hemoglobin (Hb) synthesis lead to mild to severe reduction in alpha-chain synthesis. Diagnosis of alpha-thal by examining fresh blood taken from umbilical cord is a simple and appropriate approach, while in later stages its diagnosis will be difficult and costly.

Material and Methods: This study examined the prevalence of alpha-thal gene deletion in neonates in Sari, Iran. Screening study was carried out by examining fresh blood samples obtained from excised umbilical cords of neonates born in Sari hospitals from June 2007 to March 2008. Complete blood count (CBC) was done and Hb electrophoresis and High Performance Liquid Chromatography (HPLC) were performed for detection of Hb Bart’s band. For each case two slides were stained by vital stain, 20 and 120 minutes post blood collection. Prevalence of alpha-thal was calculated and statistically analyzed (p <5%).

Results: 69 cases out of 680 (10.1%) were positive for Hb Bart’s. In 16 out of 69 positive cases (22.3%) the results of two methods, electrophoresis and slide staining were in conformity. In 53 positive cases (77%) there was no visible band in Hb electrophoresis; however Hb Bart’s was detected via vital staining method. If the ratio of mean corpuscular volume (MCV) to red blood cell (RBC) count is smaller than 23, risk of alpha-thal is 2.8 fold greater than cases with an MCV/RBC ratio below 23 (p <0.05). None of the cases were reported to be positive for Hb H disease and hydrops fetalis.

Conclusions: Considering high prevalence of alpha-thal gene deletions in neonates in Sari hospitals, it is recommended to screen newborns for alpha-thal in this city and similar areas with such a high prevalence. The sensitivity of cellulose acetate electrophoresis and HPLC methods is not adequately high to detect Hb Bart’s in all positive cases and staining and examination of peripheral blood slides stained with vital staining is necessary.

Key Words: Alpha-thalassemia, Hb Bart’s, Complete blood count, Cellulose acetate electrophoresis, HPLC.

Introduction

Alpha thalassemia (alpha-thal) is one of the most common hemoglobin (Hb) disorders in the world. Alpha-globin genes are located on chromosome 16. The majority of alpha-thal mutations are deletions, but point mutations are found as well. Since the Iranian population is a mixture of different ethnic groups, frequency and distribution of alpha-globin mutations in various regions of the country need to be clarified. These findings can contribute to a wider understanding of this disorder. The genetic disorders related to beta-thalassemia (beta-thal) are primarily point mutations inherited through a pair of genes, while genetic abnormalities in alpha-thal are mostly inherited through two pairs of genes. Due to its clinical importance, beta-thal is the more studied disorder in Iran in comparison with alpha-thal. Due to a variety of reasons different types of alpha-thal are less investigated: in case of deletion of one or two of the respective genes it lacks any clinical feature; and deletion of three genes (αα-/- αα or αα-/-αα); and deletion of three genes (-/-αα; Hb H disease) and four genes (-/-αα Hydrops fetalis) are less prevalent. Alpha-thal screening tests are important, particularly in genetic counseling for prevention of the birth of infants with beta-thal major. Deletion of one Hb alpha-chain gene is not associated with any significant clinical feature, but deletion of two genes results in microcytic hypochromia, while Hb A2 still has normal structure.

In cases of some rare types of beta-thal with normal Hb A2, this fact has to be taken into consideration.
In light of this knowledge genetic counseling followed by comprehensive laboratory examination of certain couples, of whom one or both are suspected of carrying hemoglobinopathy of one type or another, would be necessary. Until year 2005, all couples who were suspected or confirmed cases of β-thal minor, underwent counseling process and at least seven out of thousand couples were advised to take amniocentesis test. From year 2005 clinical regulations have changed in a way that following genetic counseling some cases are considered as confirmed β-thal minor couple and some other as suspected cases. For the latter group molecular genetic test is carried out to explore any deletion of alpha-thal related genes. In the past three years, the suspected group has been three fold larger than confirmed group in Mazandaran province (Bureau of Health, Mazandaran province, Iran, unpublished data). Since detection of alpha-thal gene deletion via molecular methods is expensive and few laboratories are capable of performing such tests, this approach is not suitable for an extensive public screening, but detection of Hb Bart’s (a tetramer of γ-globin chains) is a simple and inexpensive method, more appropriate for general screening. This study was aimed to determine the prevalence of alpha-thal by examining the fresh blood obtained from excised umbilical cord of neonates in Sari, Iran. Based on the findings we determined the prevalence of this type of hemoglobinopathy and suggested a justified procedure for alpha-thal neonatal screening.

**Materials and Methods**

This study was a descriptive cross-sectional assessment that was performed from June 2007 to March 2008. After receiving legal permissions, 5 to 10 ml of blood was collected from neonate umbilical cord in a tube containing 100 micro-liter of ethylenediamine tetra acetic acid (EDTA). It was tried to avoid contamination of sample with maternal blood. Complete blood count (CBC), cellulose acetate electrophoresis (in alkaline pH), HPLC and vital staining were performed on samples. For each sample two tests were done: one test 20 minutes postnatal and second one 120 minutes after birth to detect Hb Bart’s. In vital staining method Hb Bart’s is characterized by RBCs seen in a typical golf ball shape.

**Statistical Analysis**

The data were shown in descriptive statistics. Pearson’s chi-square test was carried out to correlate the results with alpha-thal diagnosis and Odd’s ratio was calculated with 95% confidence interval. Correlation was tested by Spearman’s correlation coefficient.

**Results**

In 69 out of 680 samples (10.1%), Hb Bart’s was detected. Presence of Hb Bart’s in 16 out of these 69 samples (22.3%) was confirmed by both methods, electrophoresis and staining. Finding at least one erythrocyte containing Hb Bart’s in a slide was the criteria for considering a case positive. In 53 cases (77%) there was no visible band for Hb in the electrophoresis result, but Hb Bart’s was detected in slides stained with vital staining. None of the cases were identified as having Hb H disease or hydrops fetalis.

The correlation between MCV and positive cases of alpha-thal (according to vital staining for Hb Bart’s) was statistically significant ($p < 0.05$) (Table I). The chances of having alpha-thal in a patient with an MCV smaller than 100 femtoliters (fl) is 2.97 fold higher than a patient with MCV above 100 fl ($p < 0.05$). In addition, in-

<table>
<thead>
<tr>
<th>MCV</th>
<th>Electrophoresis method</th>
<th>Vital staining method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>alpha-thal negative</td>
<td>alpha-thal positive</td>
</tr>
<tr>
<td>MCV &gt; 100</td>
<td>75</td>
<td>25.8</td>
</tr>
<tr>
<td>MCV &lt; 100</td>
<td>25</td>
<td>74.2</td>
</tr>
<tr>
<td>Total:</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Prevalence of hemoglobin alpha-Chain gene deletion in neonates in North of Iran

Deletion of four Hb alpha-chain genes is fatal and results in severe anemia and death of embryo or newborn. Types and frequency of alpha-thal mutations are different from one region to other and the spectrum of these mutations is specific for each population. Iran is located in a region with high prevalence of thalassemia and therefore identification of types and prevalence of its respective mutations are of critical importance. Neyshabouri and Abbasi-Moheb reported the frequency of -alpha 3.7 single gene deletion with typical microcytic hypochromic anemia to be 31.6% in Iranian population. This form of deletion is the most common one. They couldn’t find the second common type of mutation (-alpha4.2) in this population. In Iran the majority of the cases of two gene deletions are in the form of alpha-/alpha- and even if both parents are deletion carriers it will not result in the fatal form in the child and the most severe case will be an offspring with Hb H disease. Generally an Hb H patient does not need frequent blood transfusions. For a definitive diagnosis of alpha-thal, molecular methods, namely Gap-polymerase chain reaction (Gap-PCR), PCR-denaturing gradient gel electrophoresis (PCR-DGGE), PCR-single strand conformation polymorphism (PCR-SSCP), amplification refractory mutation system-PCR (ARMS-PCR) have to be used. All these methods are time consuming and costly. Rugless et al examined 103 samples of freshly collected umbilical cord blood to detect Hb Bart’s as a definitive marker of the presence of alpha-thal. They used HPLC method as a quantitative approach and reported Hb Bart’s to be 0.5% to 11.5% of the total of Hb molecules. This study showed that Hb Bart’s screening by examining fresh blood collected from umbilical cord is an effective method to evaluate any disturbance in globin chain synthesis, particularly in populations with high incidence of alpha-thal.

**Table II.** Distribution of cases according to blood cell indices, alpha-thalassemia diagnosis and detection methods, Sari, Iran, 2007-2008.

<table>
<thead>
<tr>
<th>Detection method</th>
<th>Alpha-thal diagnosis</th>
<th>RBC (10^6) Mean</th>
<th>S.D.</th>
<th>MCV (fl) Mean</th>
<th>S.D.</th>
<th>Hb (g/dl) Mean</th>
<th>S.D.</th>
<th>RDW* Mean</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoresis</td>
<td>Negative</td>
<td>4.064</td>
<td>0.740</td>
<td>103.2</td>
<td>5.92</td>
<td>14</td>
<td>4.21</td>
<td>17.6</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>4.47</td>
<td>1.008</td>
<td>91.7</td>
<td>9.86</td>
<td>12.8</td>
<td>2.48</td>
<td>18.4</td>
<td>2.56</td>
</tr>
<tr>
<td>Vital Staining</td>
<td>Negative</td>
<td>4.068</td>
<td>0.741</td>
<td>103.2</td>
<td>5.81</td>
<td>14.1</td>
<td>4.33</td>
<td>17.6</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>4.11</td>
<td>0.821</td>
<td>100.5</td>
<td>9.12</td>
<td>13.4</td>
<td>2.52</td>
<td>17.9</td>
<td>2.15</td>
</tr>
</tbody>
</table>

*RDW: Red cell distribution width; **S.D. Standard Deviation.

Discussion

The results of this study showed that 12% of neonates in Sari, Iran, have one or two for Hb alpha-chain gene deletions. Alpha-thal with different prevalence ranging from 1% to 98% in tropical and subtropical areas is one of the most prevalent genetic Hb synthesis disorders in the world. As an offspring inherits two copies of Hb alpha-chain gene from each of the parents there will be a variety of one to four deletions for the gene. People with only one missing gene are completely asymptomatic and even their blood cell indices are in the normal range. Identifying this deletion in post-infancy period needs molecular laboratory examinations which is a costly approach. Persons with two Hb alpha-chain gene deletions are not anemic and their Hb electrophoresis does not contain any irregular band. However, these patients have small size RBCs. Differentiating these patients from beta-thal carriers is difficult and using molecular laboratory methods is required. Three Hb alpha-chain gene deletions result in intermediate to severe anemia, similar to intermediate or major beta-thal. In Hb electrophoresis of these patients there is a detectable H band. It must be noted that in these patients Hb structure is fragile and if there is a long time gap between blood collection and laboratory testing the Hb will degrade and become undetectable. Cases of this genetic disorder are generally reported in Africa, Middle East, India, South East Asia and South of China and occasionally found in Mediterranean region. Deletion of four Hb alpha-chain genes is fatal and results in severe anemia and death of embryo or newborn. Types and frequency of alpha-thal mutations are different from one region to other and the spectrum of these mutations is specific for each population. Iran is located in a region with high prevalence of thalassemia and therefore identification of types and prevalence of its respective mutations are of critical importance. Neyshabouri and Abbasi-Moheb reported the frequency of -alpha single gene deletion with typical microcytic hypochromic anemia to be 31.6% in Iranian population. This form of deletion is the most common one. They couldn’t find the second common type of mutation (-alpha) in this population. In Iran the majority of the cases of two gene deletions are in the form of alpha-alpha and even if both parents are deletion carriers it will not result in the fatal form in the child and the most severe case will be an offspring with Hb H disease. Generally an Hb H patient does not need frequent blood transfusions. For a definitive diagnosis of alpha-thal, molecular methods, namely Gap-polymerase chain reaction (Gap-PCR), PCR-denaturing gradient gel electrophoresis (PCR-DGGE), PCR-single strand conformation polymorphism (PCR-SSCP), amplification refractory mutation system-PCR (ARMS-PCR) have to be used. All these methods are time consuming and costly. Rugless et al examined 103 samples of freshly collected umbilical cord blood to detect Hb Bart’s as a definitive marker of the presence of alpha-thal. They used HPLC method as a quantitative approach and reported Hb Bart’s to be 0.5% to 11.5% of the total of Hb molecules. This study showed that Hb Bart’s screening by examining fresh blood collected from umbilical cord is an effective method to evaluate any disturbance in globin chain synthesis, particularly in populations with high incidence of alpha-thal.
Fuchareon et al. applied HPLC method to identify Hb Bart’s and compared that with micro column technique which is an appropriate means for screening thalassemia syndromes and hemoglobinopathies during infancy stage and detects all cases of hydrops fetalis.

The results of HPLC identification of different types of hemoglobin was similar to DNA analysis performed by Sanguansermsri et al., while HPLC method is simple and faster than DNA analysis and an appropriate approach to identify alpha-thal homozygous cases. Shahriari et al. in Hb electrophoresis of 164 cases with MCV <100 fl (out of a total number of 510 samples of umbilical cord blood) identified only 12 cases as Hb Bart’s positive. All these 12 cases had MCV smaller than 93.6. In order to prevent heavy costs of specific laboratory tests to detect alpha-thal cases in infancy stage, they recommended for cases with MCV <96 fl, Hb electrophoresis to be done. Yavarian et al examined 600 umbilical cord blood samples in Bandar-Abbas, Iran, and found out that 33% of the cases contained Hb Bart’s. In our study there was no correlation between the results of HPLC method and examination of peripheral blood smears stained with vital staining. In some infants the Hb Bart’s results for both cellulose acetate electrophoresis and HPLC methods were negative. In the normal cases with MCV greater than 100 fl, reticulocyte staining did not visualize any Hb Bart’s in the slides. It seems that in the cases of deletion of one or two Hb alpha-chain genes, vital staining and examination of peripheral blood smears is a more definitive approach to identify alpha-thal positive newborns than other laboratory methods. This finding is contradictory to the results of the work of Shahriari et al. where Hb Bart’s was only detected in infants with MCV <100. In this study it was determined that newborns with a ratio of MCV/RBC smaller than 23, have 2.8 fold higher risk of having alpha-thal than the ones with an MCV/RBC above 23. Applying these criteria along with examination of peripheral blood smears stained with vital stain is a fast, accurate and inexpensive method of screening hemoglobin alpha-chain gene deletions.

Conclusion

Considering the prevalence of alpha-thal and costs of molecular laboratory methods for the pa-
tients and health system, screening neonates by examination of vital stained peripheral blood smears is recommended.

References


4) GHANEI M. Pre-marriage prevention of thalassemia: report of a 100,000 case experience in Isfahan. Public Health 1997; 111: 153-156.


