In the name of God

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Prevalence and molecular characterization of alpha-thalassemia and Detection of Hb Bart's and Hb H diseases using capillary electrophoresis among newborns in Ardabil Province

Introduction

- Apha-thalassemia is one of the most recessively congenital hemoglobin disorders in the world, which is characterized by decreased or absence of alpha globin chains production . Worldwide, up to around 5% of the general population are affected . Although the frequency of α-thalassemia in Iran is greater than it is worldwide, it is not exactly determined .

- Alpha-thalassemia syndrome results from deletion or mutation of one or both α-globin genes that are located on 16p13.3 . The phenotype of the disease is related to the number of α-globin genes deficiency as well as the type of mutation, ranging from asymptomatic phenotype to a fatal in utero disease . The loss of one (-α) and two (-) genes of α-globin as the most common cause of a-thalassemia are not associated with clinical symptoms. While, the loss of three genes of α-globin (--/-α) leads to hemoglobin H disease, which sometimes results in moderate anemia and the need for transfusion, the loss of four genes of α-globin (--/--) leads to Hb Bart's Hydrops Fetalis Syndrome, which can be fatal .

- Worldwide, more than 770 mutations have been recognized in α-globin gene clusters, and of those, more than 19 mutations documented in Iran . Gene sequencing and Multiplex Ligation-dependent Probe Amplification (MLPA) techniques can be useful for better detection of novel suspected α-thalassemia mutations . the aim of the present study was to determine the prevalence and molecular characteristic of α-thalassemia among newborns in Ardabil Province.

- The diagnosis of α-thalassemia is based on the diagnosis of Hb Bart's in infants, which indicates one or more defective or missing α-globin genes . Bart's hemoglobin level was found to be correlated with the number of defective globin α genes and is used to screen alpha thalassemia in infants . Hb Bart's has been reported to have a greater affinity for oxygen and therefore is unable to deliver effective oxygen to tissues . Hb Bart's levels in carriers of α genes range from zero to a small amount up to 1% and may even be found in people with normal α genes . Because Hb H is fast moving, and also Hb H is unstable and may not be detected by Hb electrophoresis.

- In general, previous research has shown that Hb Bart's and H in cord blood can be used as a suitable marker of alpha thalassemia, so that Bart hemoglobin levels increase in proportion to defective α genes .
- However, the effectiveness of this method has never been tested in our population. In this study, we used molecular analysis an acetate cellulose electrophoresis evaluations to detect alpha thalassemia from cord blood. Finally, our group determined the probability of α-thalassemia in Bart's and H hemoglobin screening program.

1. **Material and Methods**

- In this cross-sectional study, one thousand newborns were screened for α-thalassemia in the pediatric unit (the only research center for thalassemia cases) of Bu-Ali hospital in Ardabil city between April 2016 and March 2018.

1. Using EDTA-containing tubes, a total of 2 ml venous blood was taken from each infant for analysis during routine hematological tests (CBC, Htc, Hb, MCH and MCHC as well as ferritin) and the remainder was kept at 4˚C and sent for laboratory analysis in the first three days of life. There is a general agreement that the α-thalassemia trait is considered typical and could be present when MCV (Mean Corpuscular Volume) is below 94FL and MCH (mean corpuscular hemoglobin) is below 30 pg, respectively, except in neonates with iron deficiency

- . But in the current study, cases with MCV and MCH below 100 fL and 33 pg respectively, were referred to laboratory for measurement of serum ferritin level, acetate cellulose electrophoresis and molecular analysis. Molecular techniques have been applied to identify genetic mutations and genomic DNA was extracted from whole blood.
- Collected data were analyzed by statistical methods in IBM© SPSS© Statistics version 21 (IBM© Corp., Armonk, NY, USA).

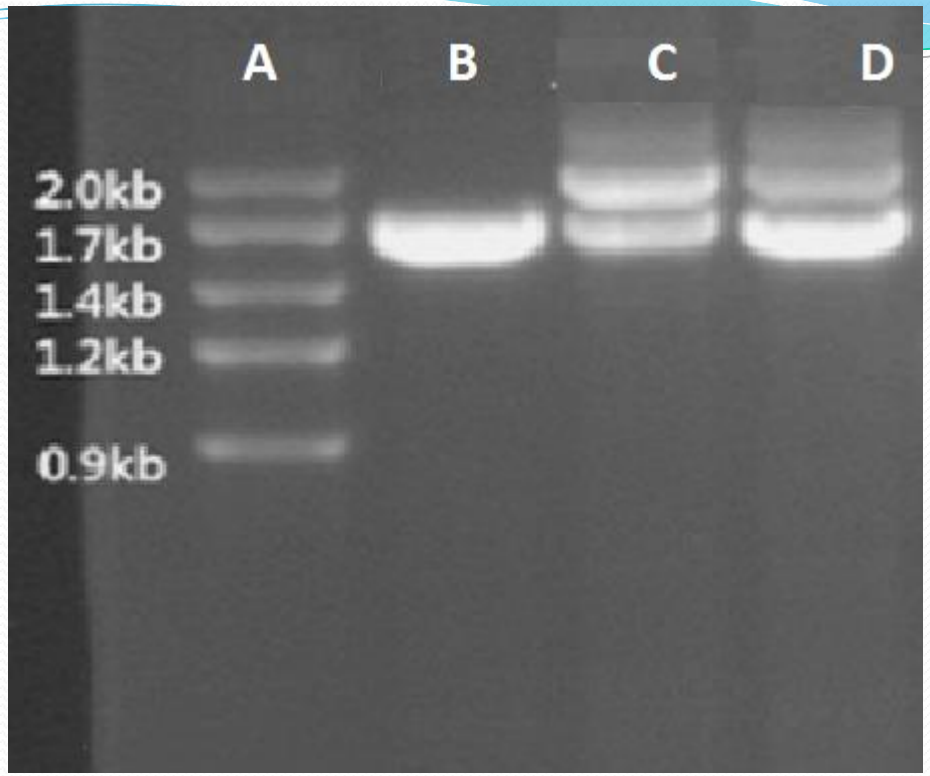

Figure 1. Multiplex PCR for screening of the most common α-thall alleles. A- Ladder marker; B- Natural genotype control (αα / αα); C- patient (α -3.7 / αα); D-positive control to remove α -3.7.

Results

- In total, 97 newborns had MCV < 100 fL and MCH <33 pg. Hb electrophoresis , serum ferritin level was carried out on all cases. Based on these results, each suspected newborn was referred for genetic analysis. In summary, we found 33 newborns with α-thalassemia based on genetic analysis . The prevalence of α-thalassemia in studied newborns was 3.3 % in Ardabil province. The most common mutation was the 3.7 single gene deletions that were found in 42.4% (14 cases) of newborn with α-thalassemia. HbH was only detected in two cases (14.7% and 15.6%) in total (6.06%) and Hb Bart's was seen only in three neonates (2.5%, 4.5% and 5.5%) in total (9.09%) (Table 1,2).

Number	RBC	Hb	Htc	MCV	MCH	MCHC	HbA	HbA2	HBF	Ferritin
33	4.50 ± 0.52	13.68±1.82	41.04 ± 5.47	92.6182± 3.70	31.20±1.63	33.78±0.82	22.36 ± 4.84	0.97 ± 0.41	73.53 ± 12.2	187.5 ± 75.5

Table 1. The mean of hematological parameters in studied samples
HbA: Hemoglobin A; HbA2: Hemoglobin A2; HbF: Fetal hemoglobin; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MCV: Mean Corpuscular Volume

Genotype	N	%	HbA2 (%)
- $\alpha^{3.7}$/ α α	14	42.4	0.8
- α3.7/ - α3.7	6	18.2	0.72
aaa anti $^{3.7}$ / aa	2	6.1	1
- α^{PA2}/ α α	1	3.0	0.9
- $\alpha^{4.2}$/ α α	4	12.2	1.75
α2 IVS1(−5nt)	1	3.0	0.8
α 2 cd19 [-G] het	1	3.0	1.5
het deletion med1	1	3.0	0.8
HET.C.427T>C at hbA2	1	3.0	2.9
del G at codon 126/Wt	1	3.0	1.8
het -a 20.5	1	3.0	1.5

Table 2. The frequency of found mutations in studied samples

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Discussion

- Although no compressive study has been performed about the prevalence of α- thalassemia in Iran , the rate of α- thalassemia carriers was estimated to be about 7.8% in the general population . Since the Iranian population is a mixture of different ethnic group, the rate of α-thalassemia can be different in different regions. Alpha-thalassemia is more prevalent around the Caspian Sea and Persian Gulf (North, and South of Iran). The rate of α-thalassemia differs from 2.7% (in East Azerbaijan; north-west of Iran) to 15.3% (in Mazandaran; northern Iran. Alpha-thalassemia is prevalent in populations in South East Asia, the Mediterranean and the Middle East. It has been reported that the prevalence of α-thalassemia in Thailand, Morocco and turkey is 30-40%, 0.96% and 0.25%-4.1%, respectively.
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- The prevalence of α-thalassemia in the current study was 3.3%. Our results are similar to the study of East Azerbaijan province, in the north-west of Iran. Data showed that $-\alpha^{3.7}$and -α4.2 are the most common mutation, causing α-thalassemia among 10,849 cases of α-thalassemia carriers in a comprehensive study of Iran . Like other regions, $-\alpha^{3.7}$/ αα and $-\alpha^{3.7}$/ $-\alpha^{3.7}$ was the most common mutation in this study, followed by -α4.2/ α α .The second most common mutation in other studies was aa/– a4.2 , poly A2 , deletion $^{--MED}$, α-5 nt .The most common mutation in the world is $-\alpha^{3.7}$ which was similar to our study results .
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- The mean of MCV and MCH was 92.62±3.7 and 31.20±1.63, respectively. So, by using these cut-off points (MCV<94 and MCH<30), a large number of cases are not detected.

- The values of MCV and MCH are strongly affected by the number of α-genes, cases with one α gene deletion have slightly decreased of these parameters and can be overlapped with normal values .
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- The mean of HbA2 and HbF were 0.97±0.4 and 73.53±12.3, respectively. HbH was only detected in two cases (14.7% and 15.6%) and Hb Bart's was seen only in 3 neonates (2.5%, 4.5% and 5.5%). The level of HbA2 in newborns with α-thalassemia carrier is normal or lower. Especially in HbH disease, it can drop to less than 2% (94% of cases in our study had HbA2 below 2%).

- Remarkably, the mean HbA2 level in this study was 0.97±0.41. HbA2 levels in infants with thalassemia carriers are normal or slightly lower, which can distinguish α-thalassemia from thalassemia in particular. In Hb H disease, HbA2 levels can be reduced to less than 1 .Normal or low HbA2 levels combined with decreased mean body hemoglobin (MCH) (<33pg) and mean cell volume (MCV) (<100fl) may be a good chance to detect α-thalassemia carriers .

. Alpha-thalassemia carriers can be identified by detection of Hb Bart’s in newborns The level of Hb Bart’s is found in a large number of newborns with α-thalassemia and it is correlated with the number of defective α-globin genes . In this study, Hb Bart’s was detected only in three cases (9.09%) and the majority of cases were negative. It is important to note that the absence of Hb Bart's does not exclude the presence of α-thalassemia in newborns (especially in mild α-3.7/αα interactions) and diagnosis can only be confirmed by the molecular analysis .

- Hb Bart's disease is not accurately diagnosed in infancy because it disappears quickly after birth. Therefore, determining the amount of Hb Bart's after infancy is not reliable . On the other hand, Hb Bart's levels have been shown to vary between different ethnic groups .

- Hb H and Hb Bart's Hb are also fast moving, appearing on electrophoresis. As a result, some reports indicate that these two parameters are unstable and may not be detectable by conventional methods .Therefore, it seems that molecular analysis of is necessary to confirm α-thalassemia.

- In this study, only five cases (15.15%) were detected by Hb Bart's and also Hb H by electrophoresis. Therefore, most infants with α-thalassemia are missed when electrophoresis alone is used. In some normal infants, Hb Bart's can be detected in about 0.5-1.5% of cases .Various factors may play a role in the variability of Hb Bart levels, and among them, the amount of γ-β globin change may play an important role .

- Our findings show that all infants with two alpha gene defects hade elevated Hb Bart , while a large proportion of single-defect alpha gene carriers dose not show traceable Hb Bart.

- Previous studies have shown that the -α4.2 allele causes a considerable synthesis imbalance of α-/non-α-globin chains and the considerable production of the γ chain in patients than the -α3.7 allele. According to such results, the level of Hb Bart in newborns with -α3.7 was 0.2 ± 0.5 was, while in newborns with -α4.2, the level of Hb Bart was represented in 0.3 ± 0.7. Therefore, our group guessed that a small amount of Hb Bart related to the -α3.7 allele is not technically reliable, while the α4.2 allele is reliably detectable due to the high level of hemoglobin Bart's.

Conclusions

- Results of this study showed that, the prevalence of α-thalassemia was 3.3% in Ardabil province. The most common mutation causing α-thalassemia in this study was $-\alpha^{3.7}/\alpha\alpha$ (42.4%). Although the evaluation of Hb Bart's and Hb H are direct methods for the diagnosis of α thalassemia , only a few cases of Alpha- thalassemia in this study were detected by Hb Bart's and Hb H (15.15%) in acetate cellulose electrophoresis and most of them were missed. Therefore molecular analysis in suspected infants is necessary to confirm α-thalassemia.

Thanks For your Attention