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ORIGINAL ARTICLE

Evaluation of the Molecular basis of *KLF1* Gene in Iranian Thalassemia individuals with borderline hemoglobin A2

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ABSTRACT
HbA2 level is a reliable diagnostic measurement for determination of beta-thalassemia carriers. Increased level of HbA2 is caused by either a mutation in B-globin gene or molecular dysfunctions occurred outside the B-globin cluster which could be attributed to the genes responsible for B globin cluster regulation such as KLF1(Krueppel- Like Factor 1). KLF1 encodes a transcriptional factor that normally acts as a transcriptional repressor for delta-globin gene and switches it off. Therefore, any defects in KLF1 gene cause HBD gene activation followed by raised expression of HbA2 . The goal of this study was to evaluate the role of KLF1 in individuals suspicious for thalassemia with borderline HbA2 level (3.3-3.9) . In this study, 60 healthy controls and 30 non-affected subjects with borderline HbA2 were recruited from southern part of Iran. Based on our results, there were no specific mutations in cases with borderline HbA2 Therefore, KLF1 gene may not be associated with this condition. It is assumed that either mutations in the promoter region of delta-globin gene or other transcriptional factors playing a role in its regulation may be the reason for this status. Further studies on different ethnical populations are required to confirm these results.
Key Words: *B-Thalasemia, HbA2 ,Krueppel-Like Factor 1(KLF1).*

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INTRODUCTION

Thalassemia is definitely an inherited autosomal recessive disease. In thalassemia, the genetic defect results in reduced rate of synthesis of among theglobin chains which makeup hemoglobin. Reduced synthesis of one of many globin chains causes the synthesis of abnormal hemoglobin molecules, and as a result causes the anemia which will be the characteristic presenting symptom of the thalassemia [1,2].

Thalassemia is just aquantitative problem of not enough globins synthesized, whereas sickle-cell disease (a hemoglobinopathy) is just a qualitative problem of synthesis of a non-functioning globin. Thalassemia usually result in less than production of normal globin proteins, often through mutations in regulatory genes [3].Hemoglobinopathies imply structural abnormalities in the globin proteins themselves [4]. Both conditions may overlap, however, since some conditions which cause abnormalities in globin proteins (hemoglobinopathy) also affect their production (thalassemia). Thus, some thalassemia is hemoglobinopathies, but the majority is not. Either or these two conditions could cause anemia [5, 6].

The illness is especially prevalent among Mediterranean peoples, and this geographical association was responsible because of itsnaming: *Thalassa* is Greek for the sea, *Haema* is Greek for blood. The thalassemia are classified based onwhich chain of the hemoglobin molecule is affected. In α thalassemia, production of the α globin chain is affected, during β thalassemia production of the β globin chain is affected [7].

The estimated prevalence of thalassemia is 16% in individuals from Cyprus, 3-14% in Thailand, and 3-8% in populations from India, Pakistan, Bangladesh, Malaysia and China. Additionally there are prevalence in descendants of individuals from Latin America, and Mediterranean countries (e.g. Spain, Portugal, Italy,

Greece and others). A surprisingly low prevalence has been reported from people in Africa (0.9%), with those in northern Africa having the greatest prevalence, and northern Europe (0.1%) [8, 9].
Generally, thalassemia is prevalent in populations that evolved in humid climates where malaria was endemic, but affects all races. Thalassemia is particularly related to Arab-Americans, individuals of Mediterranean origin, and Asians [10].
Untreated thalassemia Major eventually contributes to death usually by heart failure, therefore birth screening is extremelyimportant. B-Thalassemia cardiomyopathy assumes on two phenotypes. Dilated phenotype: Left ventricular dilatation and impaired contractility exist.Restrictive phenotype: Restrictive left ventricular filling occurs along with pulmonary hypertension, and failure. The pathophysiology of β-Thalassemia cardiomyopathy is multifactorial, with myocardial iron overload and immunoinflammatory processes being the predominant mechanisms [11, 12].
All Thalassemia patients are prone tohealth complications that involve the spleen (which is usuallyenlarged and frequently removed) and gall stones. These complications are generally prevalent to thalassemia Major and Intermedia patients [13].
Two major form of genetic factor, globin gene mutations and KLF1 gene mutation have already been reported to be linked to borderline HbA2. The globulin gene defects including mind B + thalassemia mutations co-inherited game and beta thalassemia, beta promoter mutation, alpha heterozygote, Alpha globin gene triplication and some globin variant could possibly be within about 20% of individual with borderline HbA2 [14,15].
The KLF1 gene mutations where first described because the genetic basis of the in (lu) blood group phenotype [16]. Report of the KLF1 mutations related tohereditary persistence of fetal hemoglobin (HPFH) and congenital dyserythropoieticanamia (CDA) soon followed [17, 18] in 2011, Persue *et al*. first identified six different KLF1 mutations in 52 of the 154 people who have borderline Hba2 and normal MCV and MCH [19], indication that the KLF1 mutation was among thereasons of borderline HbA2 in Italian. In Chinese, however, the genetic basis of borderline HbA2 remains poorly understands.

MATERIAL AND METHODS

Sample and Patients

The study was performed on 30 patients with borderline hemoglobin A2 and 60 healthy controls. The patients' samples were randomly taken from south of Iran. The control group was chosen from random participants whose health was confirmed by medical diagnostic.

DNA Extraction

DNA samples of both case and control group were extracted using Salting Out based on manufacturer's instructions. Nanodrop was used to evaluate the purity and concentration of genomic DNA. The reactions prepared in two tube containing 1 ng/ml forward primers, 1 ng/ml reverse primers, 6ml distilled water and 12.5 µlTaq DNA Polymerase 2x Master Mix Red. KLF1 gene was used as primer gene. The principle source of gene sequence information was taken from NCBI website. The initial denaturation step was carried out for 15 min at 94 °C, followed by second denaturation step at the same temperature for 20 seconds. The PCR cycling conditions was done for 45 sec at 45 °C, followed by 30 cycles of 45 sec at 72 °C. PCR product was run on a 2% Arose gel in 0.5× TBE buffer and visualized on a Gel Documentation System using Gel Red dye.

Patients and controls

This study included 30 patients with borderline hemoglobin A2 diagnosed in south of Iran. Blood sampling was performed after an informed consent either at the diagnosis.
Fifty healthy blood donors were used as controls.Genomic DNA was amplified by polymerase chain reaction (PCR) with congruous primers.

Primer

Four primers were designed according to a Primer Blast program at NCBI for each part of KLF1 gene.

Table 1: Primer designed for KLF1 gene

Primer name	Primer	TM	The size of the product
Exon 1-F	TTACCCAGCACCTGGACCCT	67.8°C	576 bp
Exon 1-R	GAACCTCAAACCCCTAGACCACC	67.6°C	
Exon 2-F-a	GTGTCCAGCCCGCGATGT	69.9°C	714 bp
Exon 2-R-a	CCGGGTCCCAAACAACTCA	68.2°C	
Exon 2-F-b	CCGAGATCTGGGCGCATA	68.7°C	663 bp

Exon 2-R-b	GCCCTCTGCAACCCTTCTTC	67.2°C	
Exon 3-F	TGCGGCAAGAGCTACACCA	68.1°C	538 bp
Exon 3-R	CTTGTCCCATCCCCAGTCACT	67.4°C	

PCR
The KLF1 genotyping was performed base on the Touch down. The Thermal cycling conditions for GAP-PCR were as follows in Figure1. For The PCR We Used 20 µL Sample: 1 µL Forward Primer, 1 µL Reverse Primer, 6 µL Diluents' Water, 2 µL DNA 50 ng/ml, 12.5 µL Master Mix.
Table 2:PCRprogramusedfor touch down PCR of KLF1 exon A gene

Primary Denaturation	Denaturation	Annealing	Extension	Cycles No	Final Extension
95°C 5 min	95°C 30sec	*70°C 30sec	72°C 1min	10 Cycles	-
-	95°C 30sec	57.4°C 30sec	72°C 1min	20 Cycles	72°C 10min

Table 2: PCR program used for touchdown PCR of KLF1 exon 2A gene

Primary Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles No.
95°C 5min	95°C 1min	64°C 30sec	72°C 1min	72°C 10min	35

Table 3: PCR program used for touchdown n PCR of KLF1 exon 2B gene

Primary Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles No.
95°C 5min	95°C 1min	54°C 30sec	72°C 1min	72°C 10min	35

Table 4: PCR program used for touchdown PCR of KLF1 exon 3 gene

Primary Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles No.
95°C 5min	95°C 1min	64°C 30sec	72°C 1min	72°C 10min	35

Gel Electrophoresis
The electrophoresis was carried out using 1%GelRedstained agarose gel, at 85V for 45 min We Use Horizontal Electrophoresis Cell with TBE Buffer (PH=8.3) , Ladder Were Used 50bp DNA Ladder (JenaBioscience) After electrophoresis, the amplified PCR products were Perceive under U. V. light
Statistical analysis
Statistical analyses were conducted using with the SPSS software (Statistical Package for Social Sciences) version18, was used to test the association between two categorical variables or to detect difference between two or more proportions.

RESULTS
We genotyped and analyzed 30 patients with borderline hemoglobin A2, and 60 healthy controls, for the KLF1 exon 1,2,3 gene.KLF1 exon 1,2,3 gene frequencies were in equilibrium in patients and controls(Figure 1). After investigation of all sequences KLF1 exon 1,2,3 and comparison of it with GenBankNG_013087.1 sequence through Chromas software ,there were no change in exon of KLF1 gene of individuals studied in this research.
Patients did not showed an extensively increased frequency of the KLF1 exon 1,2,3 compared with controls. Thus the KLF1 exon 1,2,3 would confer a slightly increased risk of developing late onset hemoglobin A2in Iranian population.

As a result, Carriers of the hemoglobin A2 were at a slightly but significantly increased frequency in patients compared with controls. Both groups of healthy controls, , did not had similar gene frequencies, suggesting that KLF1 exon 1,2,3 gene is not related with borderline hemoglobin A2thalassemiain Iranian population (Figure 2).Based on our results, there were no specific mutations in cases with borderline HbA2Therefore, KLF1 gene may not be associated with this condition. It is assumed that either mutations in the promoter region of delta-globin gene or other transcriptional factors playing a role in its regulation may be the reason for this status.

Figure 1:All sequences KLF1 exon 1,2,3 and comparison of it with GenBankNG_013087.1 sequence through Chromas software

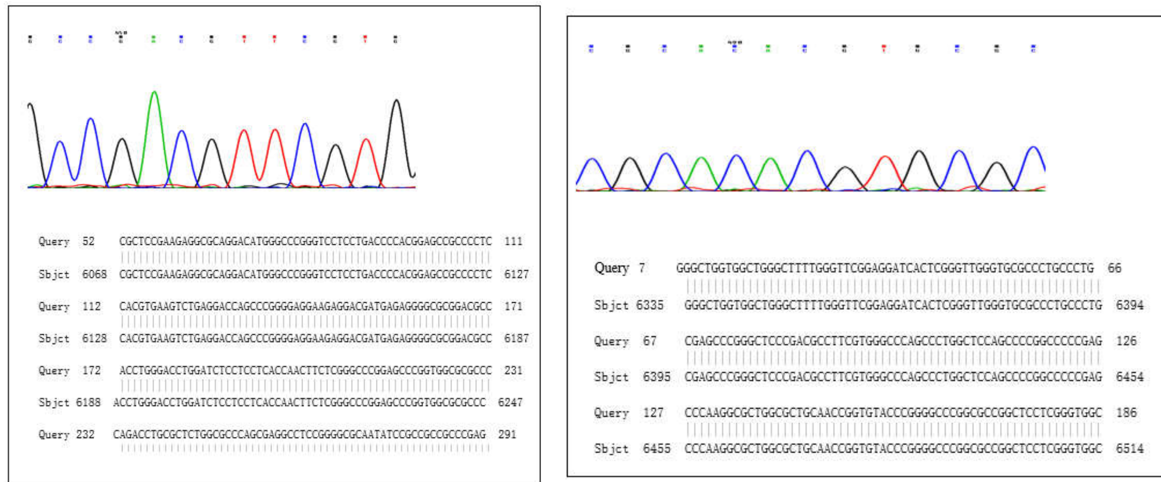
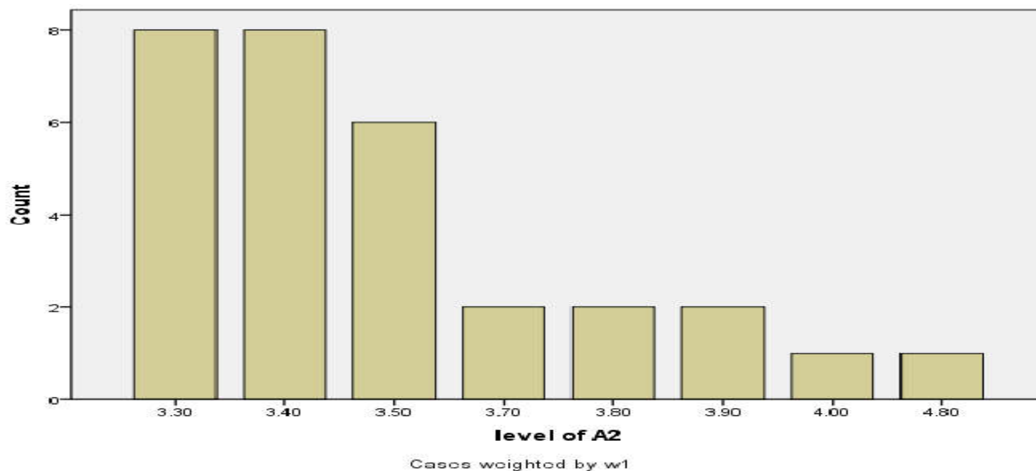


Figure 2: Borderline HbA2amount in 30 patients with borderline hemoglobin A2 in research



DISCUSSION

There is growing evidence that the KLF1 gene plays a part in the pathogenesis of borderline hemoglobin A2. Several lines of evidence suggest that KLF1 gene may play a part in borderline hemoglobin A2. In accordance with this, an increased frequency of the allele among patients with borderline hemoglobin A2has not been seen in this study.The aim of this study was to estimate the role of KLF1 in individuals suspicious for thalassemia with borderline HbA2 .In this study, 60 healthy controls and 30 non-affected subjects with borderline HbA2 were recruited from southern part of Iran. Based on our results, there were no specific mutations in cases with borderline HbA2.This study analyzed different populations, and did not find an increased frequency of the allele. As a result, we did not find any association between KLF1 gene and borderline HbA2 in our population. However, the difference between patients and controls was significant in Iranian population, and the fact that a similar result has been found in different populations suggests that KLF1 gene is may truly involve in the development of borderline HbA2thalassemia.

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