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Association between the Ssp1 loci of intron II beta globin gene with Beta Thalassemia-traits

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Original Article

Abstract

BACKGROUND: Thalassemia is a common hereditary disorder of hemoglobin synthesis. In this study, the polymorphism of the site of Ssp1 intron II of β-globin gene was investigated in patients with thalassemia minor who referred to laboratory centers in Sanandaj and Marivan townships in Iran.

METHODS: This was an analytical cross-sectional study. Initially, the questionnaires were completed to obtain the data. Based on complete blood count (CBC) and hemoglobin electrophoresis, carriers were diagnosed. Deoxyribonucleic acid (DNA) extraction was performed by the salting out method from all blood samples. The polymorphism of the Ssp1 loci of intron II beta globin gene was evaluated using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method with the effect of Ssp1 restriction enzyme on all DNA samples.

RESULTS: The results showed that of the 200 individuals or 400 chromosomes examined, 166 individuals (78 and 88% in cases and controls, respectively) were positive for SspI position and 34 individuals (22 and 12% in cases and controls, respectively) were negative. Additionally, statistical analysis suggested no significant relationship between these two groups in SspI position (OR = 0.483, P = 0.189).

CONCLUSION: The polymorphism of SspI locus in this case study is informative of Kurdish population.

KEYWORDS: Beta-Thalassemia; Ssp1 Loci; PCR-RFLP; Kurdish Population

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Introduction

Thalassemia is one of the most common genetic diseases in the world that is inherited as autosomal recessive. The disease is common in the Mediterranean, Middle East, and South-East Asian countries.[1] 1.5% of the world's population (80-90 million) are carriers of β-thalassemia, and more than 56,000 births per year lead to babies with thalassemia major worldwide, most of whom being in developing and poor countries.[2] Beta-thalassemia has a high prevalence in Iran and the national program for preventing β-thalassemia major has been in place since 1997. In this program, couples are screened for pathogenic mutations of beta-globin gene before marriage, and if both persons are carriers, pre-marital and pre-pregnancy counseling are performed.[3-8]

The highest prevalence of beta-thalassemia carriers in Iran is 10% in north of the country, border of the Caspian Sea, and south of the Gulf region and the Oman Sea. This rate in other parts of the country is estimated as 4%-8%.[4] More than 95% of all β-thalassemia mutations are spot mutations in the β-globin gene and the rest of the type of other mutations. The studies carried out in the last two decades in different provinces of the

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country indicate the dispersion of mutations in terms of type and frequency, due to genetic-ethnic heterogeneity.[4-7] The IVSI-5 mutation is the most common mutation in south east provinces including Sistan and Baluchistan and Kerman, however the mutation is less frequent in other parts of Iran. The mutations of IVSI-110, IVSI-1, Fr8-9, and C36-37 in Khuzestan, IVSII-1 in Mazandaran, and IVSII-1, C36-37, and IVSI-5 in Isfahan have the highest frequency.[1-10]

In previous reports from western and northwestern provinces of Iran, the most frequent mutations are IVSII-1, Fr8-9, IVSI-1, IVSI-110, and C36-37.[9,10] More than 200 different mutations known for beta gene have been reported in over 60 Iranian patients. Considering that Iran has a high population with a combination of different ethnic groups, determining the frequency and distribution of mutations in different parts of the country seems necessary.[4] However, due to the diversity of the population, immigration, and the domination of different ethnic groups throughout history, many mutations has not been reported. More than 40 different mutations in the β-globin gene have been reported in Iranian populations, but in some regions of Iran, mutation remains unknown in 10-20% of thalassemia minor cases.[11]

Regarding the location of the restriction enzymes in the exon and intron regions of the beta-globin gene, in addition to detecting mutations by direct sequencing, restriction enzymes and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques are also used to identify carriers of thalassemia. The locations of RFLP have been identified around the beta-globin gene, most of which do not have known polymorphism in the population of different regions of Iran. Early studies carried out in the Mediterranean and African populations show that a polymorphic position in nucleotide 666 in intron II of the β-globin gene is identifiable by the SspI restriction enzyme. Akhavan Niaki et al. investigated the frequency of polymorphisms of SspI loci in intron II in β-globin gene in Mazandaran population.[12] Their results showed that of the 422 chromosomes examined, 20.6% were negative for SspI. Negative positions were approximately equal to that of healthy and mutated chromosomes.[12]

This loci (Avall in intron II of beta-globin gene) has been reported with a significant polymorphism in the β-globin gene in Iranian population.[13] Consequently, in the present study, association between the Ssp1 loci of intron II beta globin gene with Beta Thalassemia-trait was investigated in 100 individuals with minor thalassemia and 100 healthy controls in Kurdistan population.

Methods

Sampling: In this analytical cross-sectional study, 100 patients with thalassemia minor were selected from Sanandaj, Marivan, and Saqez laboratories. The blood indexes of all specimens were measured by the automatic system (SYSMEX XS800I, Japan). From all subjects, hemoglobin electrophoresis was performed using alkaline PH acetate cellulose gel. In all patients, mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were less than 82 fl and 25 pg, respectively, and hemoglobinA2 (HbA2) was > 3.5%. In addition, 100 subjects with the same age and spatial distribution as well as MCV more than 82 fl, MCH more than 25, and hemoglobin A2 < 3.5% were selected as healthy controls. The individuals who were willing to participate in the study were selected after completing the consent form and observing the ethics of medical research plans. 5 cc of venous blood was taken from each subject and inserted in tubes containing ethylenediaminetetraacetic acid (EDTA) and kept at a temperature of 20 °C.

Molecular Experiments

Deoxyribonucleic acid (DNA) Extraction: The

DNA of the blood samples was extracted by the salting out method according to the corresponding protocols. Therefore, 5 ml of the blood sample of each patient was poured into the test tubes containing 5 mg/l of EDTA and slowly blended. Then, 5 ml of cold-buffered lysis [white blood cell (WBC) lysis] containing tris, EDTA, and NaCl was added to each sample at various concentrations. The blended samples were centrifuged at 3000 rpm for 10 minutes. After completion, the centrifugation of the supernatant was discarded and added to the clot residual of 5 ml of phosphate buffered saline (PBS) and again centrifuged at about 3000 rpm for 10 minutes. These steps continued until the white mass was completely white. After completion, the last step of the supernatant was discarded and added to the mass of the remaining white blood cells, 1 ml of cold distilled water (autoclaved), and then the contents of the tube were transferred to a 1.5 ml microtube. The tubes were centrifuged for 1 minute at about 5,000 rpm. The supernatant was discarded and added to each 600λ tris buffer and mixed. Then, 100λSDS10% and 50λ proteinase K (1 mg/ml) was added to each tube and after mixing the microtubes and place the shaker at 55 °C for one overnight. The samples were digested to one third of their volume of saturated saline solution (NaCl 5 M) and, after gentle mixing, were centrifuged at 12000 rpm at a cold temperature for 10 minutes. The supernatant was transferred to another microtube and added to the same volume of isopropanol alcohol stored at -20 °C, and then the microtube was shaken to appear DNA strands and then centrifuged at 12000 rpm at a cold temperature for 2 minutes. The supernatant was discarded and the DNA was washed three times with 75% ethanol and each time the microtube was centrifuged at 12000 rpm and the cold temperature for 2 minutes. At the last step, after removing the supernatant, the microtubes were returned to the drying paper and allowed to completely dry the DNA. To each microtube, 100 λ distilled water was added to the autoclave, allowing DNA to be completely dissolved and a uniform solution was obtained. The DNA extraction was determined on agarose gel 1% and then the isolated DNA was placed in separate microtubes and stored at -20 °C.

PCR-RFLP: Determination of genotypes was carried out by the PCR-RFLP method by the China3 and China4 primers (Table 1), which are part of the exon 3 and intron 2 beta-globin gene (Figure 1). PCR was performed in final volume of 20 µl using Sinagene PCR kit. The termocycler program is presented in table 2. To assay the correct replication, the PCR products were loaded on agarose gel 1.5% and quality of the bands was determined.

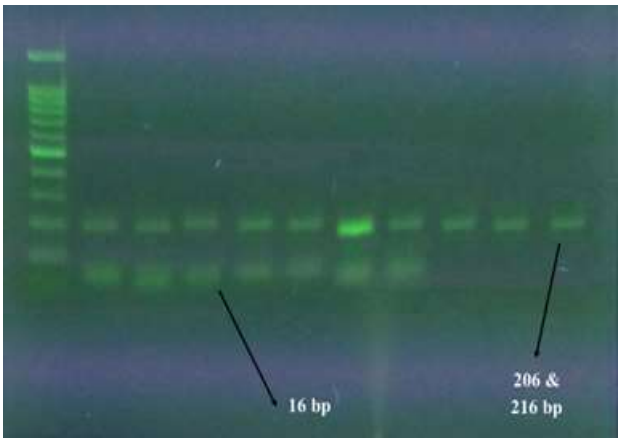

Figure 1. Enzymatic digestion of PCR products by Ssp1, the Polymerase chain reaction (PCR)-proliferated pieces are 216 bp and 206bp without cutting. In the presence of the restriction enzyme with cut positions, it results in three pieces 200, 16, and 206 bp.

Table 1. Specifications and sequencing of China3 and China4 primers

DNA Oligo Name	Oligo ID.	Sequence	GC%	NO, Bases	Tm
China3	161026J2Go11/2	GTGTACACATATTGACCAAA	35	20	45.6
China4	161026J2Ho12/2	TTGCACGACCAGACACACGA	55	20	53.8

Table 2. Polymerase chain reaction (PCR) proliferation conditions

Gene	Cycling condition				
beta-globin	Initial denaturation	Denaturation	Annealing	Extension	Final extension
	C°95 10 Minutes	C°95 60 Seconds	C°55 45 Seconds	C°72 60 Seconds	C°72 7 min
	Repeated for 38 cycles				

In order to cut the position, the SspI enzyme was selected, which is detected as ATT^AAT. If this sequence and cut is present, it was marked with + and in case of lack of it and conversion of special sequence to SspI to GTT AAT sequence due to mutation in nucleotide 666, it is shown with-sign. The PCR-proliferated pieces are 216 bp and 206bp without cutting. If the restriction enzyme had cut positions, it would result in three pieces of 200, 16, and 206 bp. Then the PCR products were affected by 10 units of SspI enzyme at 37 °C at one overnight and then the digested products were loaded on 3% agarose gel and their genotypes were determined (Figure 1).

Statistical Analysis: Data analysis was performed using software POPGENE1.32 and SPSS (version 20, IBM Corporation, Armonk, NY, USA) at the significance level of $P < 0.05$.

Results

This study was performed on 200 individuals, including 100 cases with thalassemia-trait and 100 healthy controls. Figure 1 shows the image of the agarose gel 3%, the polymorphic position, and also how to determine its genotype compared to ladder and size pieces as described above.

The analysis of results show that of the 200 individuals or 400 chromosomes examined, 166 individuals (78 and 88% in cases and controls, respectively) were positive (+) for SspI position and 34 individuals (22 and 12% in cases and controls, respectively) were negative (-) (Table 3 and Figure 2).

Table 3. Frequency of + and - SspI sites in beta thalassemia and healthy controls

Groups	N (%)	Locus
Beta thalassemia carrier	78 (78)	+
Beta thalassemia carrier	11 (22)	-
Healthy	88 (88)	+
Healthy	12 (12)	-

Besides, statistical analysis was performed by logistic regression for these sites between the carrier and healthy groups and there was no significant relationship between these two groups in SspI position in beta-globin gene (Table 4) (OR = 0.483, P = 0.189).

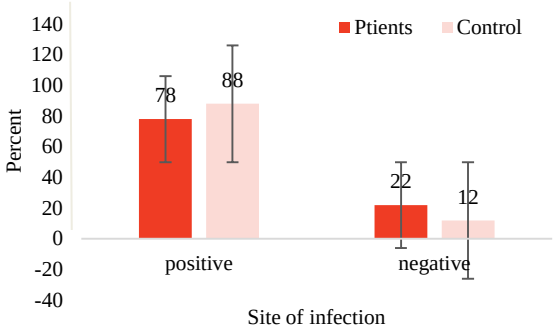

Figure 2. Frequency of position + and – SspI position in subjects studied

Discussion

More than 95% of the total β-thalassemia mutations in the world are spot mutations in the β-globin gene and a small percentage of other mutations. The studies carried out in the last two decades in different provinces of the country indicate the dispersion of mutations in terms of type and frequency, due to genetic-ethnic heterogeneity.[3]

Table 4. Logistics regression output for data analysis

Variables in the Equation		B	S.E.	Wald	df	Sig.	Exp (B)	95% CI for EXP (B)	
								Lower	Upper
Step 1a	RFLP	-0.727	0.553	1.726	1	0.189	0.483	0.164	1.430

a. Variable(s) entered on step 1: RFLP: Restriction fragment length polymorphism; CI: Confidence interval

More than 200 different mutations known for beta gene have been reported in over 60 Iranian patients. Several mutations that are responsible for β-thalassemia in Iran are of Iranian, Mediterranean, Kurdish, Turkish, Egyptian, Tunisian, Asian-Indian, Indian, and African-American backgrounds. Some mutations such as IVSII-1 (G-A) appear in most tribes, while others, such as Codon36/37 (-T), are particularly specific in Lorestan province.[4] The codon 8/8 (+ G) mutation has been introduced among the Kurdish population in Kurdistan province and West Azerbaijan in terms of common mutations.[4] According to a recent report issued by the United Nations over the past two decades, Iran has hosted many people who have often arrived from Iraq and Afghanistan. Iran has a high population with a combination of different ethnic groups, indicating the frequency and distribution of mutations in different parts of the country.[1-6]

Statistical analysis was performed by logistic regression for these sites between the carrier and healthy groups and there was no significant relationship between these two groups at SspI position in the beta-globin gene. This study is the first report in Kurdistan province. The results showed that the negative status in the thalassemia minor was higher than that of healthy people. A total of two studies have been conducted in this regard, one in Iran and another abroad. In a study by Braun et al. on African and Mediterranean populations, the frequency of polymorphism in the SspI position was reported to be 14%.[14] In another study by Akhavan Niaki et al. in Mazandaran, 211 thalassemia carriers were examined. Of the 422 chromosomes examined, 20.6% were negative for SspI. Negative sites were approximately equal to healthy and mutated alleles (11.9 and 14.3%, respectively).[12] In the present study, 22% of the negative SspI site was almost near the prevalence of this polymorphism in Mazandaran. The polymorphic positions in the population genetics and population diseases are the most informative. Due to the distance of the locus from the mutation site and also the elimination of the possibility of false results in terms of recombination and statistical analysis, the frequency above 10% of a polymorphism loci is worth examining for traceability of its alleles.[13-14]

Most of the polymorphic locations of the β-globin gene cluster are located outside of the β-globin gene, and as a result, there is a new combination probability, with some of them being of low frequency.[12] Thus, using in-gene sites such as Ssp1 with the presence of polymorphism in the population is necessary. The AvaII polymorphic position in intron II of the β-globin gene has previously been investigated by researchers in the Iranian population.[12-13] Regarding the prevalence of polymorphism in this loci and the lower cost of SspI by half the cost of the AvaII enzyme, the polymorphism of the SspI site in intron II of the β-globin gene is an appropriate place for the genotype to be investigated indirectly (RFLP).[13-14] Moreover, since the frequency of absence of SspI locus in the population of Kurdistan province is more than 10% (22%), the negative SspI status is a good indication for its pursuit. Due to the importance of diagnostic accuracy in prenatal diagnostic tests and the possibility of human and laboratory errors, it is recommended to use two different methods to determine fetal genotype.

Conclusion

In cases where mutation of the beta-globin gene is not detectable, in which the type of parent mutation has been identified, the polymorphism of the SspI site can be used as a complementary method for confirming the fetus genotype.

Conflict of Interests

Authors have no conflict of interests.

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