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Original Article

Alpha-Hemoglobin Stabilizing Protein Gene Polymorphism (rs4499252 A/G) and its Association with Beta-Thalassemia Major in Iraqi Patients

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Abstract

Beta thalassemia (β-thalassemia) major is a genetic disorder of hemoglobin production that results in a diminished rate of synthesis of one or more of the globin chains causing variable degrees of anemia. Alpha-hemoglobin-stabilizing protein (AHSP) is a specific alpha-globin factor that affects the severity of the disease in patients with β-thalassemia. A recent study was conducted to investigate the polymorphism in the *AHSP* (rs4499252) gene and its association with β-thalassemia in Iraq. Blood samples were obtained from 90 β-thalassemia patients and 60 healthy individuals as a control group in the Wasit Center for Hereditary Anemia from August 2020 to January 2021. After DNA extraction from the whole blood, to determine the genotype of the *AHSP* gene, the High-Resolution Melt (HRM) Real-Time PCR was used. The results showed a significant increase ($P<0.05$) in genotype GG (wild type) of the SNP (rs4499252) in β-thalassemia patients, compared to the control group. On the other hand, genotype AA was significantly higher ($P\leq0.05$) in β-thalassemia patients than in the control group, while the genotype GA showed a non-significant difference ($P<0.01$) between β-thalassemia patients and the healthy controls. The results also showed that the *AHSP* expression is a biomarker of hemoglobin H disease severity, and the A allele was more frequent in β-thalassemia patients than the G allele in Iraqi patients.

Keywords: AHSP, β-thalassemia, Iraq, Polymorphism, Real-Time PCR

1. Introduction

Beta thalassemia (β-thalassemia) is an inherited autosomal recessive disease that causes different degrees of anemia. The molecular defects are caused by point mutations or small deletions that reduce or eliminate globin chain synthesis (1). The unmatched -Hb is toxic to itself and other cellular components resulting in inefficient erythropoiesis and hemolysis (2).

The Alpha-Hemoglobin Stabilizing Protein (AHSP) is an erythroid scavenger protein that attaches the subunit of the hemoglobin molecule quickly and reversibly preventing precipitation and subsequent hemolysis, and it is primarily expressed during erythropoiesis and can reversibly bind to αHb G and H helices of free αHb forming a complex (AHSP-αHb) (3). Moreover, it maintains αHb available for incorporation with βHb to form functional HbA1 ($\alpha2\beta2$) which is called adult HB (4).

The AHSP is the special molecule chaperone that binds to the chains of hemoglobin and provides a possible compensatory mechanism for the deleterious effects of a-globin precipitation in erythroid precursors

(5, 6). The human *AHSP* gene is located at chromosome 16 (16p11.2) spreading over 952 bases possessing three exons, two introns, and one untranslated region (UTR) being the first exon and another UTR at the end of exon 3. The translation initiation and the termination codons are located at the 2nd and 3rd exons, respectively. *AHSP* gene encodes a small protein molecule known as AHSP (11.84 kDa) possessing 102 amino acids which are expressed at the highest level in the bone marrow. AHSP contains about 70% α-helices and can exist in *cis* and *trans* isomeric forms (7). Accordingly, based on these observations, it has been proposed that alleles altering the levels or function of AHSP might account for some of the clinical variability observed in patients with thalassemia; therefore, this study aimed to investigate the association of polymorphism of AHSP with β-thalassemia in Iraqi patients.

2. Materials and Methods

2.1. Patient Selection

This study was conducted from August 2020 to January 2021. Signed written consent was taken from each individual participating in the study, and a specific questionnaire form was used for subject information.

The study was designed to be a prospective case-control study. The samples selected included β-thalassemia major patients from Wasit Centre for Hereditary Anemia diagnosed as β-thalassemia major. A total of 90 β-thalassemia major patients within the age range of 5-22 years were included in the study. Thalassemia patients were diagnosed using Hb electrophoresis, complete blood count, and serum ferritin level by the center's physicians. It should be noted that 60 healthy individuals were selected as controls.

The patients who received therapy for thalassemia, those with hepatitis B and C, as well as splenectomized cases were excluded from the study.

2.2. Blood Sample Collection

The blood samples (3 ml) were obtained from each individual in each group by venipuncture using disposable syringes, and the samples were then placed directly into EDTA anticoagulant tubes for Hb electrophoresis, complete blood count, and molecular analysis.

2.3. DNA Extraction from Blood Samples

The DNA extraction from the whole blood of both patient and control groups was conducted using the protocol in *EasyPure*® Blood Genomic DNA Kit (Transgene, China) according to the company's instructions. Subsequently, DNA concentration and purity were measured by Nanodrop. The nucleic acid concentration and purity ratio were also automatically calculated by the NanoDrop spectrophotometer, and the results were ranged within 30-120 ng/µl and 1.8-2, respectively.

2.4. Primer Preparation

After dissolving the lyophilized primers (Table 1) in nuclease-free water according to the manufacturer to make a stock solution with a concentration of 100 µM for each primer, a primer working solution was prepared and stored at -23˚C. Dilution of 10µL of primers stock solution in 90 µL of nuclease-free water yielded a working solution with a concentration of 10 µM, which was stored at -23˚C until use.

Table 1. Sequence of primers of AHSP rs4499252 high-resolution melt used in this study

AHSP rs4499252 high-resolution melt	
Rivers	CCATAGCATTTCGAGCCTGG
Forward	CAAACCAGGGGCATGTTCC

2.5. High-Resolution Melt Real-Time PCR

To detect the genetic variation in the *AHSP* gene which was chosen for this study, one single nucleotide polymorphism (SNP) (AHSP rs4499252) was selected to investigate its association with the patient problems. SNP detection was achieved by HRM Real-Time PCR. A Rotor gene (Qiagen) was used to perform qPCR-HRM according to the program in table 2, followed by an HRM analysis with 0.2˚C ramping from 65˚C to 95˚C. HRM Master Mix Synthetic SNP sequences were tested using duplicates and

contained EVA-Green. To detect allelic differences, qPCR-HRM was performed in triplicate on synthetic controls. Following that, normalized melting curves and differential curves were obtained using the HRM tool included in the integrated software (Rotor gene 4.4). The components of quantitative Real-Time PCR are presented in table 3.

Table 2. Thermal profile of HRM genotyping of AHSP

Step	Temperature	Duration	Cycles
Enzyme activation	95ºC	5 min	1
Denature	95ºC	8 sec	
Annealing	60ºC	15 sec	40
Extention	72ºC	15 sec	
HRM	65-95	0.2 sec for 1degree	1

Table 3. Components of quantitative Real-Time PCR used in the AHSP experiment

Components	*20 µl rxn*
Eva green master mix	10.0
Nuclease free water	5
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
DNA	3
Total volume	20

2.6. Statistical Analysis

The Statistical Analysis System (SAS) (8) was used to detect the effect of different factors on the study parameters. The least significant difference (LSD) test and ANOVA were used to significantly compare the means. The Chi-square test was also utilized to significantly compare between percentages (0.01 probability) in this study (8).

3. Results and Discussion

The genetic variation in the *SNP* (AHSP rs4499252) gene was chosen to investigate gene polymorphism and association with a β-thalassemia major in Iraqi patients. Genomic DNA is separated and purified from the whole blood using a DNA purification kit (Transgene, China) for the patient and control groups. All specimens show bands, which referred to the genomic DNA on the gel electrophoresis (Figure 1).

The nucleic acid concentration and purity ratio were automatically calculated by the NanoDrop spectrophotometer, and the results were ranged within 30-120 ng/µl and 1.8-2, respectively. HRM Real-Time PCR is a new and homogeneous technique that allows genomic researchers to examine genetic variations (SNPs, mutations, and methylations) in PCR amplicons. This method is beyond the capabilities of traditional melting curve analysis by allowing for a much more detailed and information-rich study of the thermal denaturation of double-stranded DNA than ever before. HRM is used to measure the dissociation (melting) behavior to characterize nucleic acid samples. Samples can be distinguished based on their sequence, length, GC content, and strand complementarity. SNPs and even single-base changes can be easily identified (9). To detect genetic variation, the *AHSP* gene was chosen for this study. The SNP (AHSP rs4499252) was selected to investigate its association with the β-thalassemia major, and its detection was achieved using HRM Real-Time PCR. DNA samples of the two study groups were genotyped of the *AHSP* gene (rs4499252). The resulting output of the thermocycler of the two genotypes is shown in figure 2. The process of HRM analysis is shown in figure 3.

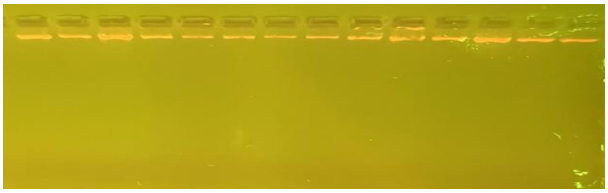

Figure 1. Gel-electrophoresis of DNA extracted from the whole blood sample, the fragments were fractionated by electrophoresis on 1% agarose and 1X TBE (1h /90v).

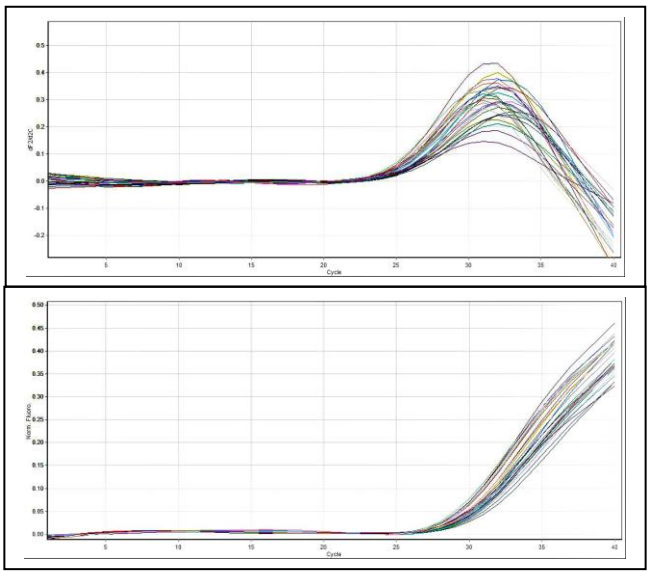

Figure 2. Result output of the thermocycler of the two genotypes by HRM analysis

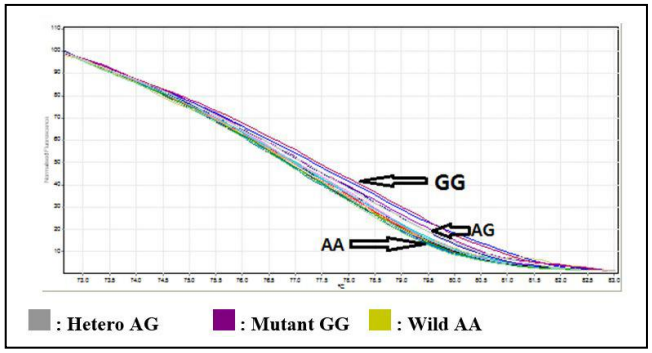

Figure 3. Resulting output of HRM for the genotype and polymorphism of AHSP

The AHSP is a molecular chaperon that can reversibly bind to free αHb subunits to form AHSP-αHb preventing αHb precipitation and keeping αHb available for incorporation with βHb when sufficient βHb is available in the circulation (7). Genotype and allele frequency distribution (Table 4) showed the results of genotype and allele frequency of *SNP1 AHSP* gene rs4499252 A/G in patients and the control group. It seems that the genotype frequency of AA shows a significant difference between patients (n=45, 50%) and healthy controls (n=38; 63.33%). Moreover, a non-significant difference in the genotype AG was observed in the patients (n=29; 32.22%) and healthy controls (n=19, 31.67%); however, the results revealed a significant increase in the genotype GG in the patients (n=16; 17.78%) and the control group (n=3; 5%), whereas a non-significant difference was noted between A and G allele (P<0.01).

As related to the GG genotype (wild-type), the frequency of this genotype was significantly higher in the β-thalassemia group than in the control group. However, the GA genotype frequency was non-significantly different (P<0.01) in the β-thalassemia and the control groups. In addition, AA genotype frequency was significantly (P<0.01) higher in the β-thalassemia group, compared to the control group. Regarding the A allele (79% for control and 66% for patients) and G allele (21% for control and 34% for patients), it seems that the difference of genotype and allele frequency was significant between β-thalassemia patients and controls.

The results in table 5 show the compression of the genotype and allele frequencies of the *AHSP* gene polymorphism (rs4499252) between patients and controls. The allele frequency of the co-dominant gene GG had a significant increase in patients (n=16; 17.8%), while it was obtained at 3.4% in the controls (n=2) (P=0.021). Moreover, the dominant gene was AG+GG in patients (n=45; 50.0%), while it was 62.5% in the controls (n=26), and it revealed a significant decrease in patients, compared to the control group (P=0.42).

According to the results, the GG allele frequency was 17.8% in patients (n=16), whereas it was 3.4% in the controls (n=2), and it shows a significant increase in patients, compared to the control group (P=0.033). The results of G allele frequency also revealed a significant increase in patients (n=61; 33.9%), compared to the healthy controls (n=28; 23.4%) (P=0.049). Table 6 summarizes a significant difference in the AA gene between patients and controls regarding the age groups of 5-10 years and 11-18 years; however, a non-significant difference was noted for the AG and GG genes at all age groups in the study.

A study on genotype variation based on gender showed a non-significant difference between patients and controls in this regard, except for the AA gene in males (Table 7).

Table 4. Genotype and allele frequency of the *SNP1 AHSP* gene (rs4499252) A/G in the control and patient groups

SNP1: rs4499252 A/G	Control No. (%)	Patients No. (%)	*P*-value	Odd ratio (95% CI)
Genotype				
AA	38 (63.33%)	45 (50.00%)	0.0392 *	1.00
AG	19 (31.67%)	29 (32.22%)	0.833 NS	1.288 (0.76-2.07)
GG	3 (5.00%)	16 (17.78%)	0.0411 *	4.503 (2.16-7.11)
Allele	**Frequency**			
A	0.79	0.66	--	--
G	0.21	0.34	--	--

* ($P \leq 0.05$)

Table 5. Comparison of the genotype and allele frequencies of the *AHSP* gene polymorphism (rs4499252) between the patient and control groups

AHSP polymorphism rs4499252	Frequencies (%)		*P*-value	Odd ratio (95% CI)
	Control group (n=60)	Patient group (n=90)		
Codominant				
AA	56.6% (n=34)	50.0% (n=45)	---	1.00 (Reference)
AG	40.0% (n=24)	32.2% (n=29)	0.79	0.91 (0.4-1.8)
GG	3.4% (n=2)	8% (n=16).17	0.02	6.0 (0.1-2.8)
Dominant				
AA	56.6% (n=34)	50.0% (n=45)	---	1.00 (Reference)
AG+GG	62.5% (n=26)	50.0% (n=45)	0.42	1.3 (0.6-2.5)
Recessive				
AA+AG	96.6% (n=48)	82.2% (n=74)	---	1.00 (Reference)
GG	3.4% (n=2)	8% (n=16).17	0.033	5.3 (0.1-2.3)
Allele				
A	(92) 67.6%	66.1% (119)	---	1.00 (Reference)
G	23.4% (28)	33.9% (61)	0.049	1.6 (0.9-2.8)

Table 6. Genotype of *SNP1 AHSP* gene/rs4499252 A/G in the controls and patients with different age groups

Age groups (year)	SNP1: rs4499252 A/G	Control No. (%)	Patients No. (%)	*P*-value
5-10 (year)	AA	3 (5.00%)	22 (24.44%)	0.0092 **
	AG	3 (5.00%)	11 (12.22%)	0.267 NS
	GG	0 (0.00%)	6 (6.67%)	0.397 NS
11-18 (year)	AA	35 (58.33%)	23 (25.56%)	0.0056 **
	AG	16 (26.67%)	18 (20.00%)	0.388 NS
	GG	3 (5.00%)	10 (11.11%)	0.402 NS

** ($P \leq 0.01$), NS: Non-Significant

Table 7. Genotype of the *SNP1 AHSP* gene/ rs4499252 A/G in the controls and patients regarding gender

Gender	SNP1: rs4499252 A/G	Control No. (%)	Patients No. (%)	*P*-value
Male	AA	23 (38.33%)	21 (23.33%)	0.0255 *
	AG	10 (16.67%)	17 (18.89%)	0.871 NS
	GG	0 (0.00%)	10 (11.11%)	0.164 NS
Female	AA	15 (25.00%)	24 (26.67%)	0.933 NS
	AG	9 (15.00%)	12 (13.33%)	0.846 NS
	GG	3 (5.00%)	6 (6.67%)	0.926 NS

* ($P \leq 0.05$), NS: Non-Significant

The alleles altering the level of function of AHSP might account for some of the clinical variability observed in patients with β-thalassemia (10). AHSP mutation leading to its reduced expression or altered function provides evidence that AHSP could modulate β-thalassemia. The association of reduced AHSP mRNA expression with a more severe phenotype was observed among individuals with identical β-thalassemia (11). AHSP mutation in β-thalassemia patients is uncommon, and to date, the association between AHSP mutation and β-thalassemia variability has not been well stabilized (12). The hypothesis is that AHSP may act as a genetic modifier in β-thalassemia (13, 14). The polymorphism in different SNP-differed polymorphisms may be due to the differentiation of the genetic population (15). The alleles altering the level of function of AHSP might account for some of the clinical variability observed in patients with β-thalassemia (10, 16).

The results of the current study are in line with the findings of a study by Viprakasit, Tanphaichitr (17) since they showed mutations (ATG-AGG) in the chain; however, the differences in these results might be due to genetic changes between the Iraqi population and Chinese. The reduced expression of AHSP was associated with a more severe phenotype among individuals with identical β-thalassemia and α-globin genotypes although no mutations or polymorphism in the gene could be implicated (18). The polymorphism of rs4499252 A/G SNP in the *AHSP* gene was associated with developing β-thalassemia disease, and the A allele was more frequent in β-thalassemia patients than the G allele in the Iraqi population.

Authors' Contribution

Study concept and design: M. A. K.
Acquisition of data: M. A. K.
Analysis and interpretation of data: B. Q. H. A.
Drafting of the manuscript: H. Q. M.
Critical revision of the manuscript for important intellectual content: M. A. K.
Statistical analysis: S. H. E.
Administrative, technical, and material support: M. A. K.

Ethics

All the study experiments were performed and approved by the Ethics Committee of the Institute of Genetic Engineering and Biotechnology for the Postgraduate Studies, University of Baghdad, Baghdad, Iraq, as well as the laboratories of Wasit Centre for Heredity Anemia.

Conflict of Interest

The authors declare that they have no conflict of interest.

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