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Original Article

HV2 fragment mutations in β-thalassemia patients and a new base pair insertion of high-altitude cases

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Abstract: Worldwide, thalassemia represents one of the most common genetic disorders. There is a prevalence of Beta-thalassemia in Kingdom of Saudi Arabia (KSA), however there is a genetic counseling availability and an existence of mandatory premarital testing policy. Few studies detect molecular mutations of thalassemia genes in different KSA governates, including Makkah, Hufuf, Qatif, and Dammam but in our peer knowledge there is no reports on high altitude Taif region. The aim of the present study is to evaluate the molecular mutation analysis of β-thalassemia gene in El Taif province (as a high-altitude area) patients of KSA and to estimate the iron overload toxicity due to thalassemia syndrome on the hotspot noncoding D-loop region (hypervariable, HV2 gene fragment) of mtDNA. Blood samples were collected from total 25 β-thalassemia patients and 25 normal control that were used for HPLC, hematological analysis and different molecular evaluations. Extracted nuclear DNA from blood sample was used to detect known mutations accompanied with β-thalassemia in other countries using PCR-ARMS technique targeting IVSII-1, IVSI-5, Codon 8/9, Cd44 and Cd5 genes' mutations. Moreover, mtDNA was used to detect point of mutation of HV2 fragment in the D-loop region using PCR-SSCP and then sequencing. Results show significant increase in the level of HbA2 and decrease of HbA in comparison to control by using HPLC. PCR-ARMS reports that all β-thalassemia patients have heterozygous alleles of wild and mutated regions with nucleotide transition/transversion of IVSI-5 (AC>AG), Codon 8/9 (CT>CC), and Cd44 (GG>GA), however no point of mutation was detected in IVSII-1 (AC>AT) Cd5 (CT>CG) genes. Moreover, PCR-SSCP shows points of mutations for β-thalassemia HV2 fragment that were confirmed by sequencing in the form of base pairs deletion, insertion and transition/transversion. For the first time, the present study reports the presence of 2 bps found in HV2 region that might be specific to KSA nations and not found in other countries. In conclusion, our results were in concurrent with other studies in the presence of specific genetic mutations in β-thalassemia patients that is accompanied with points of mutations in HV2 region of high altitude Taif governate.

Keywords: β-thalassemia, KSA, Taif governate, PCR-ARMS, PCR-SSCP

Introduction

Deficiency or the complete absence of beta globin chains' synthesis due to genetic mutations represents the main cause of beta thalassemia syndromes. β-thalassemia syndrome characterized by a group of hereditary disorders that results in major or intermedia thalassemia [1, 2].

There are about 150-200 million people carriers of the β-thalassemia gene (3% of the populations) around the world [3]. It was reported about 300 different mutations in β-globin gene with about 40 subsets of mutations that are responsible for most cases, according to population studies, worldwide [4].

β-globin gene's mutations are population specific and it is highly prevalence in Mediterranean countries, the Middle East, Central Asia, and others. In addition, it is reported in all Arab countries with different frequencies with carrier rate ranges from 1 to 11% [5]. β thalassemia is widely prevalent in Saudi Arabia, with the highest prevalence around Jubail, Qateef,

Dammam, and Hofuf provinces (Eastern provinces), Southwestern and along the coastal strip of the Red Sea [6, 7], however consanguineous marriages increase incidence more than 50% [8]. The incidence of thalassemia in KSA has dropped dramatically after the beginning of premarital screening program in 2004 [9].

It is known that mitochondria are a major target for iron toxicity due to β-thalassemia, that was observed *in vitro* and *in vivo* due to mtDNA mutation and respiratory enzymes shortage [10, 11]. Previous study hypothesized that iron overload affects calcium or zinc sequestration that in turn can lead to oxidative stress and reactive oxygen species (ROS) production inside the mitochondria and mutate mtDNA. Consequently, possible mtDNA polymorphisms accompanied with β-thalassemia disease may complicate the correlation of genotype-phenotype and in turn could affect patients' clinical outcomes [30]. The noncoding region D-loop assigned as a hot spot region, especially hypervariable fragment (HV1, HV2 and HV3), for mtDNA mutation due to cancers, aging, diseases, or any other stress [12].

The present study was aimed to explore molecular mutation analysis of β-thalassemia gene using polymerase chain reaction-amplification refractory mutation system (PCR-ARMS) technique in a group of β-thalassemia patients in El Taif province (as a high-altitude area) of KSA. Moreover, we aimed to examine the toxicity occurred on mitochondria due to iron overload toxicity due to thalassemia syndrome and high altitude by assessing mtDNA mutation in the hotspot noncoding D-loop region (hypervariable, HV2 gene fragment) using Single-strand conformation polymorphism (SSCP) technique and then sequencing.

Materials and methods

Blood samples collection

Fifty human blood samples of control (25) and β thalassemia patients (25) were collected in EDTA tubes from Taif central blood bank and Hematology clinics in King Abdulaziz Specialist Hospital, TAIF. This retrospective study was approved by the Research Advisory Council of the Directorate of Health Affairs-Taif, Research and Studies Department, IRB registration number with KACST, KSA: HAP-02-T-067, Approval number: 142, at Date: 3/12/2018.

HPLC, capillary electrophoresis and CBC evaluations

All blood samples (normal and β-thalassemia patients) were analyzed on high performance liquid chromatography (HPLC) VARIANT II and then by the Capillarys 2 electrophoresis system (CE) for hemoglobin quantification/identification according to manufacturer. Moreover, different complete blood count (CBC) parameters were done for the same blood samples in Taif central blood bank and Hematology clinics in King Abdulaziz Specialist Hospital, KSA.

Molecular evaluation

nDNA and mtDNA extraction: Nuclear and mitochondrial DNA were extracted from blood sample, at the same time, manually by salting out according to combined techniques of Beckman et al. [13] and Ahmad et al. [14]. Blood (1 ml) was mixed with 1 ml of cold low salt buffer (100 mM Tris-HCl, pH 7.4, 250 mM sucrose, 10 mM EDTA) until it turns transparent red. nDNA precipitated by centrifugation for 10 mins at 1500 × g, while supernatant that contains mitochondria was transferred into a new tube and centrifuged for 10 mins (10000 × g). Both nDNA and mtDNA were suspended in high salt buffer (Tris HCl 10 mM pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 0.4 M NaCl and 2 mM EDTA), and then 75 µl of 10% SDS and 1 µl proteinase K enzyme were added and mixture was incubated at 55°C for 30 mins. After protein salting out by 6 M NaCl, nDNA and mtDNA were precipitated by cold ethanol, dried, and dissolved sterile deionized water.

PCR-ARMS for nDNA: Optimization of PCR-ARMS reaction was done after several trials until adopting the following recipe, in the same sequence, to obtain 25 µl PCR reaction mixture: 7.5 µl Deionized water, 2 µl nDNA sample (100 ng), 1 µl (20 pmole) internal control primer A, 1 µl internal control primer B (20 pmole), 0.5 µl common primer C or D (20 pmole), 0.5 µl wild or mutant primer (20 pmole), and finally 12.5 µl Green master mix.

Two different PCR-ARMS reactions (in two tubes) were done for each sample: One for the normal allele (using the normal primer) and the other for the mutant allele (using the mutant primer) detection. PCR-ARMS was programmed to detect the mutations of: IVSII-1, codon 8/9, codon 44, IVSI-5 and Cd5 (wild and mutated primers were shown in **Table 1**),

Table 1. Primers used in PCR-ARMS technique [15]

For all PCR-ARMS reactions	Primer A (forward)	5'-CAA TGT ATC ATG CCT CTT TGC ACC-3'
	Primer B (reverse)	5'-GAG TCA AGG CTG AGA GAT GCA GGA-3'
Common primer	Common primer C	5'-ACC TCA CCC TGT GGA GCC AC-3'
	Common primer D	5'-CCC CTT CCT ATG ACA TGA ACT TAA-3'
Primer C	IVSII-1 (AC) Wild	5'-AAG AAA ACA TCA AGG GTC CCA TAG ACT GAC-3'
	IVSII-1 (AT) Mut	5'-AAG AAA ACA TCA AGG GTC CCA TAG ACT GAT-3'
Primer C	Codon 8/9 (CT) Wild	5'-CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT-3'
	Codon 8/9 (CC) Mut	5'-CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC-3'
Primer C	IVSI-5 (AC) Wild	5'-CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC-3'
	IVSI-5 (AG) Mut	5'-CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG-3'
Primer C	Cd44 (GG) Wild	5'-AGC ATC AGG AGT GGA CAG ATC CCC AAT GG-3'
	Cd44 (GA) Mut	5'-CAG CAT CAG GAG TGG ACA GAT CCC CAA TGA-3'
Primer D	Cd5 (-CT) Wild	5'-ACC ACA GAC ACC ATG GTG CAC CTG ACT CCT-3'
	Cd5 (-CG) Mut	5'-TCA AAC AGA CAC CAT GGT GCA CCT GAG TCG-3'

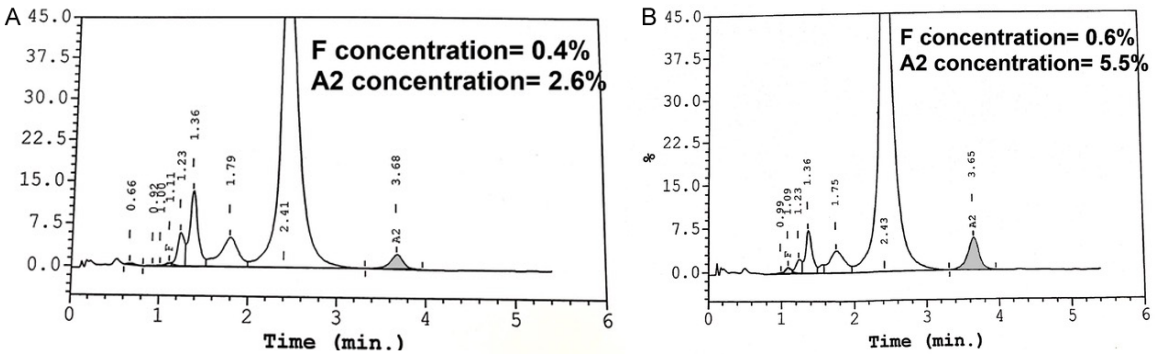

Figure 1. HPLC chromatogram of normal control (A) and beta thalassemia patient (B) showing hemoglobin elution of HbA2.

program was established as: initial denaturation at 94°C for 5 min, double stranded denaturation at 94°C for 1 min, primer annealing at 65°C for 1 min, and primer extension at 72°C for 1 min, for 35 cycles and final extension at 72°C for 10 mins [15]. The PCR-ARMS products were electrophoresed on 2% agarose gel and run at 100 V for 30 mins, and then bands are visualized under UV transilluminator.

PCR and SSCP for HV2 of mtDNA: D-Loop region of mtDNA was known as a hot spot region for various mutations, hypervariable region HV2 position from 29-408 bp was amplified to produce amplicon 380 bp. PCR reaction mixture was setup by using forward primer (5'-GGTCTATCACCCTATTAACCAC-3', 20 Pmol) and reverse primer (5'-CTGTTAAAAGTGCATACCGCCA-3', 20 Pmol) [16]. Program of PCR was programmed with initial denaturation at 94°C (5 min), DNA denaturation at 94°C (30 sec), primer annealing at 55°C (1 min), and primer extension at 72°C for 1 min for 30 cycles. And final extension at 72°C for 10 mins.

For SSCP technique: PCR product was denaturated by adding 1 × TE buffer and denaturing-loading dye (95% formamide, 4 M urea, 0.1% bromophenol blue, 0.1% Xylene cyanol FF and 0.5 µl 15% Ficoll), heated to 94°C for 5 min and then chilled on ice for 10 mins [17, 18]. The denaturated PCR samples were separated to 9% polyacrylamide gel electrophoresis (acrylamide:bisacrylamide = 19:1 v/v). Gel was excised and visualized under UV for detection of any point of mutation.

Table 2. Different CBC (complete blood count) parameters and hemoglobin fractions for normal and beta thalassemia cases represented by mean ± SD

	HbA2	HbA	Hb	RDW	MCHC	MCH	MCV	HCT	RBC	WBC	PLT
Normal	2.90 ± 0.40%	97.1 ± 1.55%	145.44 ± 19.26 g/L	12.09 ± 0.97% CV	325.92 ± 10.68 g/L	28.33 ± 2.25 pg	86.85 ± 4.68 fL	0.45 ± 0.05 L/L	5.14 ± 0.59 × 10^{12}/L	7.08 ± 2.50 × 10^{9}/L	277.04 ± 51.93 × 10^{9}/L
Patient	5.33 ± 0.58%***	93.97 ± 1.75%***	118.0 ± 19.26 g/L***	15.25 ± 4.34% CV***	313.28 ± 18.26 g/L***	20.85 ± 2.03 pg***	65.96 ± 5.62 fL***	0.41 ± 0.06 L/L***	5.99 ± 1.09 × 10^{12}/L***	5.41 ± 2.00 × 10^{9}/L***	246.00 ± 35.00 × 10^{9}/L***

In which, ***indicates $P \leq 0.001$ by comparing different patients' parameters with their corresponding in the control by using student t-test. In which, Hb, hemoglobin; HCT, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PLT, platelets; RBCs, red blood cells; RDW, red cell distribution width.

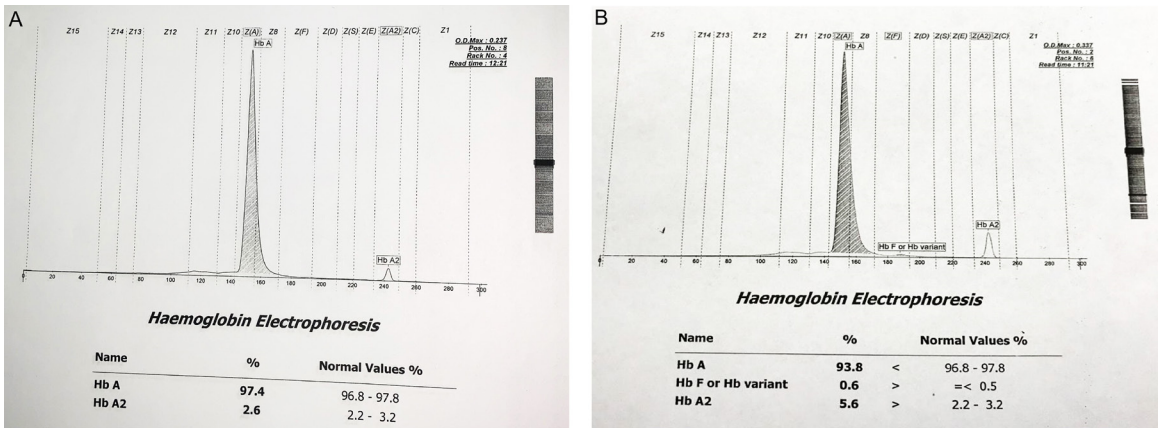

Figure 2. Capillary electrophoresis (CE) of hemoglobin for normal control (A) and abnormal increase of HbA2 and Hb variant and decrease of HbA of beta thalassemia patient (B).

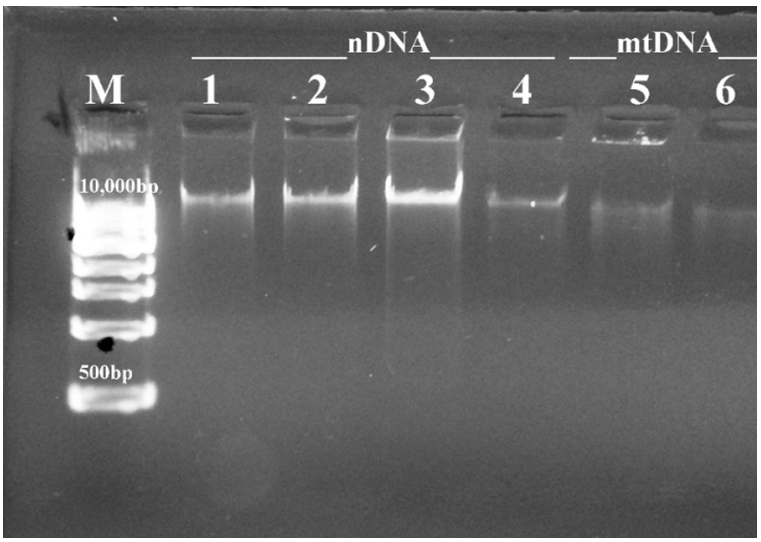

Figure 3. Agarose gel (1.5%) showing extracted mtDNA and nDNA, and M refers to high molecular DNA marker (500-10,000 bp).

Automated sequencing for mutated PCR product: PCR product was cleaned up and then undergoes automated sequencing by using BigDye Terminator v3.1 Cycle Sequencing kit using Biosystem automated sequencer (The ABI PRISM 3100 Genetic Analyzer). Sequences of mutated PCR was compared by sequence of non-mutated control PCR to detect the point of mutation.

Statistical analysis

Different CBC patients' data were compared by their corresponding control data by using student t-test using GraphPad software (GraphPad, 2017)®. In which, *** indicates $P \leq 0.001$, ** indicates $P \leq 0.01$, * indicates $P \leq 0.05$ and ns (non-significant) means $P > 0.05$.

Results

HPLC, capillary electrophoresis and CBC evaluations

Firstly, the present study used HPLC technique for identification and quantification of HbA2 and HbF. **Figure 1** represents chromatogram of HbA2 elution for normal control 2.6% (at retention time = 3.68 min) and β-thalassemia 5.5% (retention time = 3.65 min), while HbF elution for normal control 0.4% (at retention time = 1.11 min) and β-thalassemia 0.6% (retention time = 1.09 min).

Different blood cells abnormalities were accompanied with β-thalassemia patients, including lymphocytosis, neutropenia, polycythemia, anemia, microcytic RBC, microcytic PLT hypochromic, hyperchromic, monocytosis, thrombocytosis, leukocytosis, and thrombocytopenia. **Table 2** records mean ± SD of different CBC parameters and hemoglobin for normal control and β-thalassemia patients as a simple and specific tool to evaluate thalassemia disease. In which, β-thalassemia patients show a highly significant increase of HbA2, RDW and RBC and decrease of HbA, HGB, MCHC, MCH, MCV, HCT, WBC and PLT in comparison to negative normal control cases by using student t-test at $P \leq 0.001$.

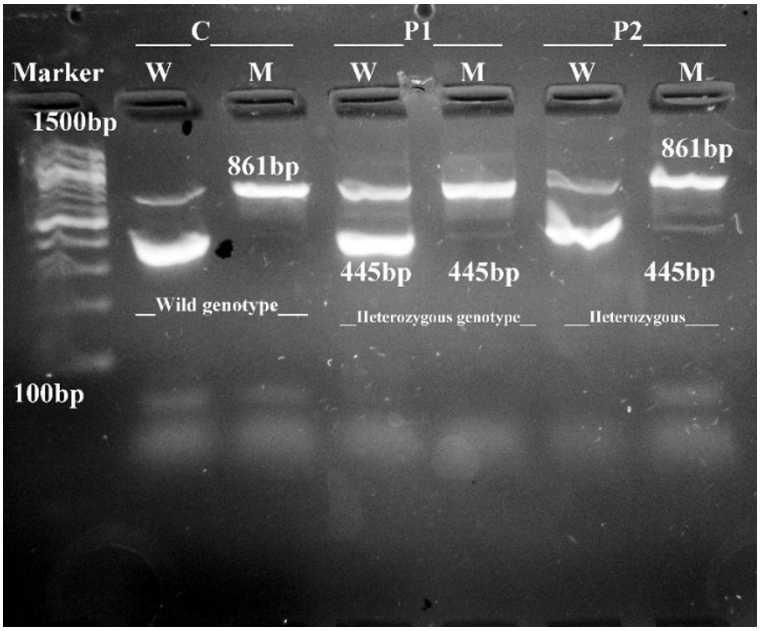

Figure 4. Polymerase chain reaction-amplification refractory mutation system products of Cd44 β-thalassemia mutation on 2% agarose gel. In which C shows normal control, P1 and P2 represent beta thalassemia patients with heterozygous genotype, and Marker represents Low molecular weight DNA Ladder (100-1500 bp), W: Wild, M: Mutant.

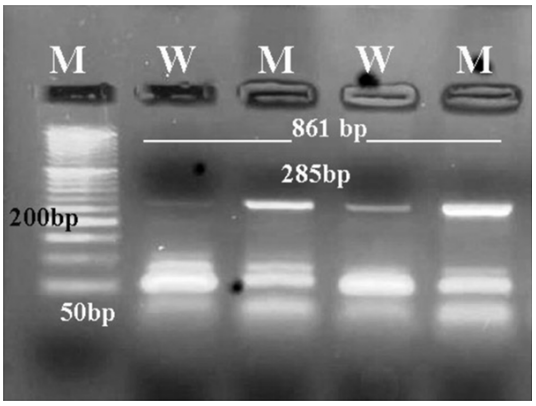

Figure 5. Representative PCR-ARMS products of IVSI-5 β-thalassemia mutation on 2% agarose gel. In which all the samples for beta thalassemia patients representing heterozygous genotypes, and 1st lane (M) represents Low molecular weight DNA Ladder (50-1500 bp), W: Wild, M: Mutant.

Representative printout of capillary electrophoresis of HbA and HbA2 in normal and β-thalassemia cases were shown in **Figure 2**, in which normal cases show normal values of HbA and HbA2 within ranges 96.8-97.8% and 2.2-3.2%, respectively. However, β-thalassemia patients show decrease of HbA (93.8%), increase of HbA2 (5.6%) and presence of HbF/Hb variant (0.6%).

Evaluate β-thalassemia gene mutation by PCR-ARMS

Figure 3 shows a successful extraction of undegraded nDNA and mtDNA for further evaluations. In which extracted nDNA was used to detect mutation in beta globin genes at different known specific sites, while mtDNA was used for HV2 fragment evaluation. The present study selects the most popular five mutations linked to β-thalassemia that is found in different countries as Iran, Turkey and inside different provinces of KSA: Cd44 (GG>GA), IVSII-1 (AC>AT), Cd5 (CT>CG), IVSI-5 (AG>AC) and Codon 8/9 (CT>CC). For each mutation, two PCR-ARMS reactions (two tubes) were done for each sample: one to detect normal allele (using the wild primer) and the other for mutant allele (using the mutant primer). In all successful PCR-ARMS reactions, 861 bp band for the internal PCR control product was detected in both wild and/or mutant samples.

Figures 4-**6** are representative PCR-ARMS products for Cd44, IVSI-5, and Codon 8/9 mutation detection on 2% agarose gel, respectively, in which all samples contain an internal control band at 861 bp (referred to faint bands with line in the expected size in gel photo). Sample C represents normal control sample contains an amplified product in wild (W) only, while all β-thalassemia patients have 2 bands wild and mutant for Cd44 (GG>GA) 445 bp, IVSI-5 (AG>AC) 285 bp and Codon 8/9 (CT>CC) 225 bp assigning individuals to heterozygous genotype. However, all normal control and β-thalassemia patients' samples have only wild alleles of IVSII-1 (AC) 634 bp and Cd5 (CT) 500 bp and absence of mutant bands IVSII-1 (AT) 634 bp and Cd5 (CG) 500 bp, indicating the absence of point of mutation in those selected genes as shown in **Figure 7**.

Detection of HV2 fragment mutation by SSCP and sequencing

PCR for D-loop HV2 fragment was successful for normal control and β-thalassemia patients

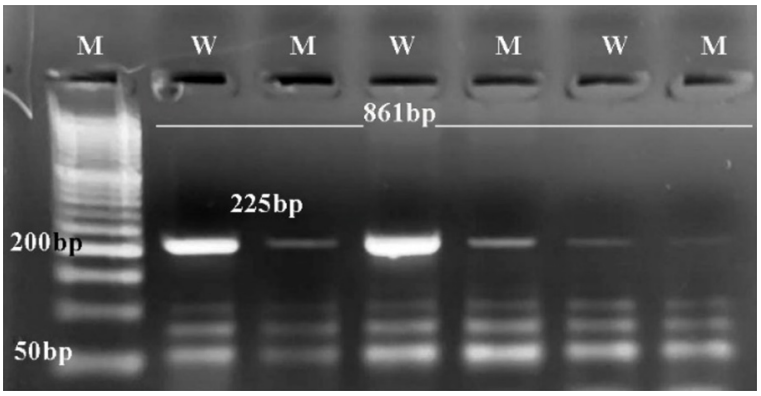

Figure 6. Representative PCR-ARMS products of Codon 8/9 β-thalassemia mutation on 2% agarose gel. In which all the samples for beta thalassemia patients representing heterozygous genotypes, and 1st lane (M) represents Low molecular weight DNA Ladder (50-1500 bp), W: Wild, M: Mutant.

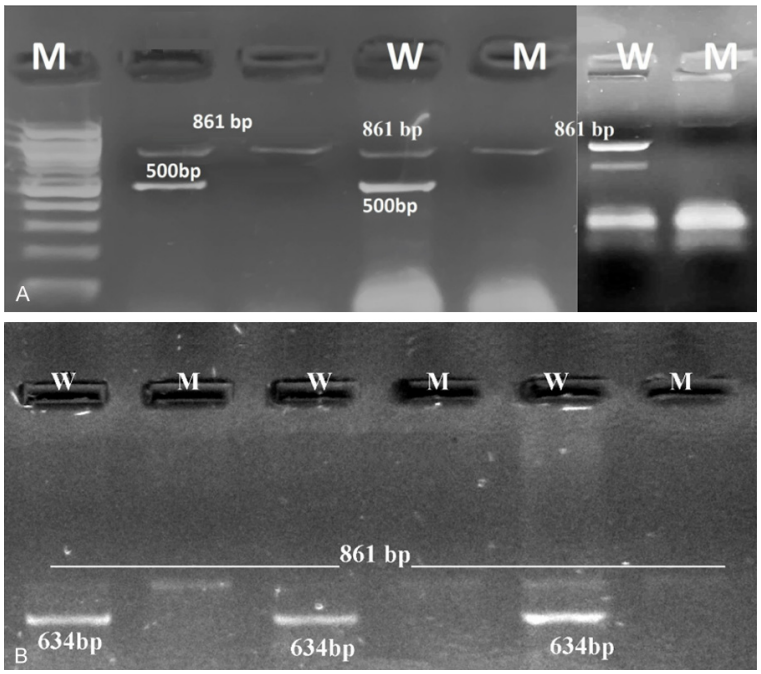

Figure 7. Representative PCR-ARMS products of Cd5 (A) and IVSII-1 (B) β-thalassemia mutation on 2% agarose gel. In which all the samples from normal control and β-thalassemia patients appear normal with wild genotypes only, and 1st lane (M) represents Low molecular weight DNA Ladder (100-1500 bp), W: Wild, M: Mutant.

with expected amplicon size about 421 bp as shown in **Figure 8A**. The primers used from Ahmad et al. [14] was designed to amplify HV2 fragment from mtDNA D-Loop at position from 8 bp to 429 bps. **Figure 8B** is a representative 9% polyacrylamide gel for PCR-SSCP pattern of HV2 fragment for normal and β-thalassemia patients that shows band shifts, extra bands below and above the normal control bands indicating points of mutations.

PCR product was subjected to sequencing to detect exact site of mutation. Two different control samples were used, one from the same collecting samples' place Taif governate as a high altitude and the other control from Jeddah governate as a low altitude place, to detect if there is a difference between controls and thalassemia patients according to different altitude. In addition, HV2 gene fragment sequence from NCBI genebank was used for comparison. Results show that sequences of β-thalassemia patients show different points of mutations represented by base pair deletion, insertion, and substitution (transition and transversion) but they are not common and differs from patient to other as shown in **Figure 9A**. Moreover, the sequences of both normal controls of high altitude (Taif) and low altitude (Jeddah) are same, however they show slight difference when comparing them with that of NCBI genebank (GenBank accession number NC_012920). All samples, either normal control or β-thalassemia patients, have a common insertion of 2 bps; 1 bp at 309-310 bps (inserted C), and 1 bp at 315-316 bps (inserted C) that is not found in NCBI genebank sequences that means it is specific to Taif/Jeddah governate nation (**Figure 9B**). We have submitted this sequence in Genbank with an accession number "MT882040" but it is not yet published online in the site.

Discussion

According to previous studies that show an agreement with the present one; Old et al. [19] report that the expected normal values for HbA2 ranges from 1.7% to 3.2% in normal cases, while carriers of β-thalassemia ranges

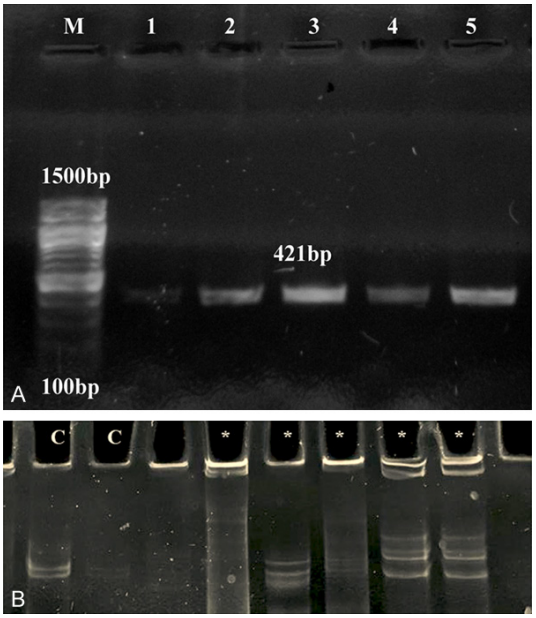

Figure 8. Agarose gel (1.5%) showing PCR product of HV2 fragment with size 421 bp (A) while M represents low molecular weight DNA marker (100-1500 bp), 1-2 represent control, while 3-5 are representative for patient HV2 PCR product. (B) Represents 9% polyacrylamide gel showing PCR-SSCP pattern for HV2 fragment of D-Loop of mtDNA. 1st lane C, represents normal control samples; * represents change of bands patterns by shift, delete or extra bands that means points of mutations for β-thalassemia patients.

from 4.0% to 7%, however (3.2%-3.8%) values are considered borderline and need further investigation. In addition, they report that the normal HbF value is usually <1.5% of total hemoglobin.

The present study agreed with Jameel et al. [20], they refer that microcytosis and hypochromia are common in iron deficiency anemia and β-thalassemia disease, however other different blood parameters could differentiate between them. In addition, mild anemia that is unresponsive to medicine was related to the asymptomatic minor thalassemia. In a previous study of Jameel et al. [20] in a Saudi Arabian Premarital Screening Program, they report low Hb and MCV values and increase in RBCs count and RDW of β-thalassemia patients. They conclude that RDW as a sign of anisocytosis represents the first index to become abnormal in iron deficiency disease [21-23]. Leung et al. [24] report a low MCV and increase of HbA2 values as a diagnostic way for β-Thalassemia carriers.

The present molecular results of PCR-ARMS were in consciences with previous research by Mashi et al. [25] on Saudi β-thalassemia patients, in which they report nineteen different mutations in all detectable (103 β-thalassemia patients) cases including IVS-II-1G>A (32%) and VS-I-5G>C (11%) and other five novel mutations (c.410G>A, c.-31C>T, c.68_74delAAGTTGG, c.316-3C>A, and c.-151C>T) were identified for the first time in Saudi population. Previously, it was reported that about 3% of the populations (~150 million people) in the world are β-thalassemia carriers [26, 27]. In addition, it seems that β-thalassemia mutations are population specific, this means that each country has its own unique and frequency mutations.

mtDNA was selected to detect any mutagenic change accompanied by β-thalassemia in high altitude area, because mtDNA was more susceptible to mutations than nDNA due to absence of complex chromatin organization protection, repair capacity shortage, and highly affected by electron transport chain due to superoxide radicals generation [28]. There are two different hypervariable regions (HV1 and HV2) present in the non-coding D-loop region, however their sequence analyses are used for forensic analyses and medical diagnosis [29].

The present results were in agreement with Jamali et al. [30], in which they investigated four mtDNA D-loop polymorphisms at nucleotides 16,069C>T, 16,189T>C, 16,319G>A, and 16,519T>C that showed significant differences between β-thalassemia patients and normal control. They suggest that iron overload in β-thalassemia patients' body could lead to oxidative stress and production of reactive oxygen species inside the mitochondria that in turn leads to mtDNA mutations.

Previously, it was reported that several human pathologies and normal aging were related to impairment of mitochondrial processes due to mutations of mtDNA, protein-coding and tRNA genes [31]. In addition, the presence of 2 bps inserted in HV2 region could be specific to KSA nation but this suggestion must be proved by broader sequencing of several samples from different governates. Several studies used control region D-loop of mtDNA as an identification tool in forensic casework or geographic regions [32, 33]. In addition, the present work used HV2 fragment as a point of study because most of mtDNA related studies scope on

A

Control 54 AGACGCTGGAGCCGGAGCACCCTATGTCGCAGTATCTGTCTTTGATTCCTACCCCATCCC 113

Beta-Th 52 AGACGCTGGAGCCGGAGC-CCCTATGTCGCAGTATCTGTCTTTGATTCCTGCCCCATCCT 110

Control 114 ATTATTTATCGCACCTACGTTCAATATTACAGGCGAACATACCTACTAAAGTGTGTTAAT 173

Beta-Th 111 ATTATTTATCGCACCTACGTTCAATATTACAGGCGAACATACTCACTAAAGTGTGTTAAT 170

Control 174 TAATTAATGCTTGTAGGACATAATAATAACAATTGAATGTCTGCACAGCCGTTTTCCACA 233

Beta-Th 171 TAATTAATGCTTGTAGGACATAATAATAACAATTGAATGTCTGCACAGCCGC-TTTCCACA 230

Control 234 CAGACATCATAACAAAAAATTTCCACCAAACCCCCCCCTCCCCCCGCTTCTGGCCACAGC 292

Beta-Th 231 CAGACATCATAACAAAAAATTTCCACCAAACCCCCCCCTCCCCCCGCTTCTGGGCACAGC 290

Control 293 ACTTAAACACATCTCTGCCAAACCCCAAAAACAAAGAACCCTAACACCAGCCTAACCAGA 352

Beta-Th 291 ACTTAAACACATCTCTGCCAAACCCCAAAAAAAAAAACCCTAACACCAGCCTAACCAAA 350

Control 353 TTTCAAATTTTATCTTTTGGCGGTATGCA-CTTTTAA 388

Beta-Th 351 TTTCAAATTTTATCTTTTGGGGGGATGCCCCTTTTAA 387

B

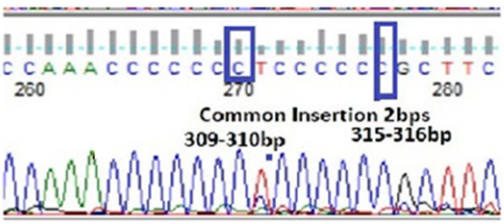

Figure 9. Alignment of HV2 PCR sequence between control normal sample of High-altitude region (Taif governate) and Beta Thalassemia patient sample (A) that shows different mutations with red colors. (B) Shows common insertion of base pair with their location written below sequences and underlined in blue color in (A).

HV1 segment rather than HV2. Amorim et al. [34] reported that it is important to be in progress with mtDNA sequence databases to cover a wider area of mtDNA typing capability to additional populations and to increase the existing databases' size.

Inclusion criteria: All thalassemia patients' samples were collected from Taif central blood bank and Hematology clinics in King Abdulaziz Specialist Hospital, TAIF as a part of premarital screening program that is mandatory done in KSA before marriage. *Exclusion criteria*: For control patients' samples with an evidence of a significant, active hematological disease and/or cumulative blood donations during clinical trials in the last three months.

Conclusions

In conclusion, our results were in concurrent with other previous studies, in which β-thalas-

semia disease could be detected in a sensitive manner by HPLC and capillary electrophoresis. In addition, reported nDNA mutations in beta globin gene is commonly found in β-thalassemia patients in other countries worldwide. Moreover, there are different point of mutations found in HV2 gene fragment of D-Loop region linked with β-thalassemia patients. For the first time in our peer knowledge, the present study reports 2 bps inserted in HV2 gene fragment that is found in Taif/Jeddah nations only and not found in the public genebank. More studies must be done to explore mechanism of point of mutations accompanied with β-thalassemia and more blood samples must be collected from other provinces for identification of specific sequence of HV2 that might be related to KSA nations only.

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Disclosure of conflict of interest

None.

Abbreviations

CBC, Complete blood count; CE, Capillary electrophoresis; Hb, Hemoglobin; HCT, Hematocrit; HPLC, High performance liquid chromatography; HV, Hypervariable region; KSA, Kingdom of Saudi Arabia; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; PLT, Platelets; PCR-ARMS, Polymerase chain reaction-amplification refractory mutation system; RBCs, Red blood cells; RDW, Red cell distribution width; ROS, Reactive oxygen species; SSCP, Single-strand conformation polymorphism; WBCs, white blood cells.

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