KLF1 gene and borderline hemoglobin A$_2$ in Saudi population

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Abstract

Introduction: Elevated HbA$_2$ (hemoglobin A$_2$) level is considered the most reliable hematological parameter for the detection of β-thalassemia carriers. However, some carriers are difficult to recognize because the level of HbA$_2$ is not in the distinctive carrier range, i.e. 4.0–6.0%; instead, some carriers have HbA$_2$ levels between normal and carrier levels, i.e. borderline HbA$_2$ (HbA$_2$ = 3.1–3.9%). Studies have shown that variations in the erythroid Krüppel-like factor (*KLF1*) gene lead to borderline HbA$_2$ in β-thalassemia carriers from various populations. The incidence of borderline HbA$_2$ in Saudis is high.
Material and methods: To confirm the influence of variations in *KLF1*, *HBA1*, *HBA2* and *HBB* genes for the reduction of the level of HbA$_2$ in Saudi β-thalassemia carriers, we performed a direct sequence analysis of *KLF1*, *HBA1*, *HBA2* and *HBB* genes from 212 healthy Saudis (88 subjects: HbA$_2$ < 3; 72 subjects: HbA$_2$ = 3.1 to 3.9; 52 subjects HbA$_2$ > 4.3).
Results: The presence of the borderline HbA$_2$ level is not specific to any type of β-thalassemia variation or β$^+$-thalassemia variations in Saudis. Two exonic (c.304T>C and c.544T>C) and two 3′ untranslated region (3′UTR) (c.*296G>A and c.*277C>G) variations have been identified in the *KLF1* gene for the first time from an Arab population. None of these four variations in *KLF1* genes are significantly associated with the Saudis with borderline HbA$_2$. α Globin genotype, $-\alpha_2^{3.7}/\alpha_1\alpha_2$, is found to be the most frequent (55.55%) among healthy Saudis with borderline HbA$_2$ compared with the other groups (HbA$_2$ < 3 = 20.45%; HbA$_2$ > 4.3 = 13.51%).
Conclusions: Further studies are necessary to determine the influence of other factors on the presence of borderline HbA$_2$ in 41.67% of Saudis.

Key words: β-thalassemia carrier, borderline HbA$_2$, *KLF1* gene, Saudi Arabia, variations, *HBB* gene, *HBA1* gene, *HBA2* gene.

Introduction

The Al-Qatif and Al-Ahsa regions in the Eastern Province of Saudi Arabia are well known for their high prevalence of sickle cell anemia and β-thalassemia. Patients homozygous for β-thalassemia require long life

blood transfusion therapy that imposes a tremendous impact on the health institutes in the area. To reduce the rate of incidence of β-thalassemia major, the health authorities implemented a premarriage screening policy to identify subjects with β-thalassemia trait [1–5]. Elevated HbA$_2$ (hemoglobin A$_2$) level (4% to 6.0%) is considered the most reliable haematological parameter used for identification of patients heterozygous for a β-thalassemia variation [6–9]. However, in some instances the level of HbA$_2$ is not within the range which infers the carrier status of β-thalassemia [5, 7–9]. These carriers usually have a borderline level of HbA$_2$ in the range of 3.1–3.9%, which makes it difficult to infer the presence or absence of the β-thalassemia variation. Therefore, molecular identification of the β-thalassemia carrier variation has been proposed for inclusion in the pre-marital screening program to reduce the incidence of newborns with β-thalassemia major [4, 7–9]. However, screening of a large population using molecular diagnosis is proven to be challenging and expensive especially in rural areas.

Recent studies have shown coinheritance of a variation in the erythroid Krüppel-like factor (EKLF or KLF1) leads to a borderline HbA$_2$ level in β-thalassemia carriers [7–12]. KLF1 is a sequence-specific DNA binding factor, which is encoded by the *KLF1* gene. *KLF1* is an essential and primary transcription factor for α and β globin genes [11]. Therefore, the present study was conducted to establish the association of variations in the *KLF1* gene in our subjects with borderline

HbA$_2$ and also to shed some light on the possible effects of this variation in the *KLF1* gene on the level of HbA$_2$.

Material and methods

A total of 212 Saudi healthy individuals (88 subjects: HbA$_2$ < 3 or ranging from 0.6% to 3%; 72 subjects: HbA$_2$ = 3.1 to 3.9; 52 subjects HbA$_2$ > 4.3 or ranging from 4.3% to 7.2%) were included in the study (Table I). The study was approved by the Institutional Review Board at the University of Dammam (approval number: IRB-2013-08-030). Written informed consent was obtained from all participants. The clinical data of each subject, including the level of HbA$_2$, were obtained. Genomic DNA was isolated from the blood samples using commercially available genomic DNA (GE health care, UK). The level of HbA$_2$ was measured for all subjects using a Variant II Analyzer (Bio-Rad, USA). All the samples were subjected for the identification of β and α globin gene variations [5, 6, 13].

Direct DNA sequence analysis of the *KLF1* (forward primer: 5′-TTTATCTGGGAGGGGCATTTTTATAG-GACC-3′ and reverse primer: 5′-GTGTCCAGC-CCGCGATGT-3′), *HBB*, *HBA1* and *HBA2* genes was performed using a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) as described earlier [4, 5, 12]. Sequence analysis was carried out using DNA sequencing analysis software V5.4 (Applied Biosystems, Foster City, CA, USA) and Variobox [14]. MAFFT (Multiple sequence alignment and NJ/UPGMA phylogeny)

Table I. Hematological parameters in Saudis participating in the study

Parameter	Group		
	HbA$_2$ < 3	HbA$_2$ 3.1–3.9	HbA$_2$ > 4.3
n	88 (24 F, 64 M)	72 (16 F, 56 M)	52 (14 F, 38 M)
Age	26.51 ±11.02	30.44 ±9.68	25.8 ±12.36
Hb [g/dl]	12.99 ±1.87	12.53 ±1.72	11.74 ±1.37
MCV [Fl]	81.68 ±10.38	77.24 ±9.48	64.44 ±13.26
HbF (%)	1.49 ±3.52	1.05 ±0.73	1.69 ±2.8
Ferritin [µg/l]	132.48 ±91.02	NA	62.83 ±56.38
Iron [µmol/l]	73 ±15.1	NA	75.33 ±39.46
HbS (%)	27.65 ±21.42	29.76 ±7.95	22.94 ±28.93
HbA$_2$ (%)	2.77 ±0.34	3.41 ±0.3	5.5 ±0.44
Height [m]	1.69 ±0.17	1.71 ±0.06	1.63 ±0.25
Weight [kg]	87.36 ±26.9	92.21 ±21.11	66 ±32.74
BMI [kg/m^2]	29.83 ±7.43	31.27 ±6.46	23.2 ±6.21

Hb – hemoglobin (Hb) concentration, MCV – mean corpuscular volume, HbF – hemoglobin F, HbS – hemoglobin S, HbA$_2$ – hemoglobin A2.

version 7 was used for the multiple sequence alignment of the *KLF1* gene sequences [15]. Three-dimensional (3D) homology modeling of wild and mutated (NM_006563.3:c.304T>C and

NM_006563.3:c.544T>C corresponding to S102P and F182L) KLF1 proteins was carried out as described earlier using different kinds of bioinformatics tools [16, 17].
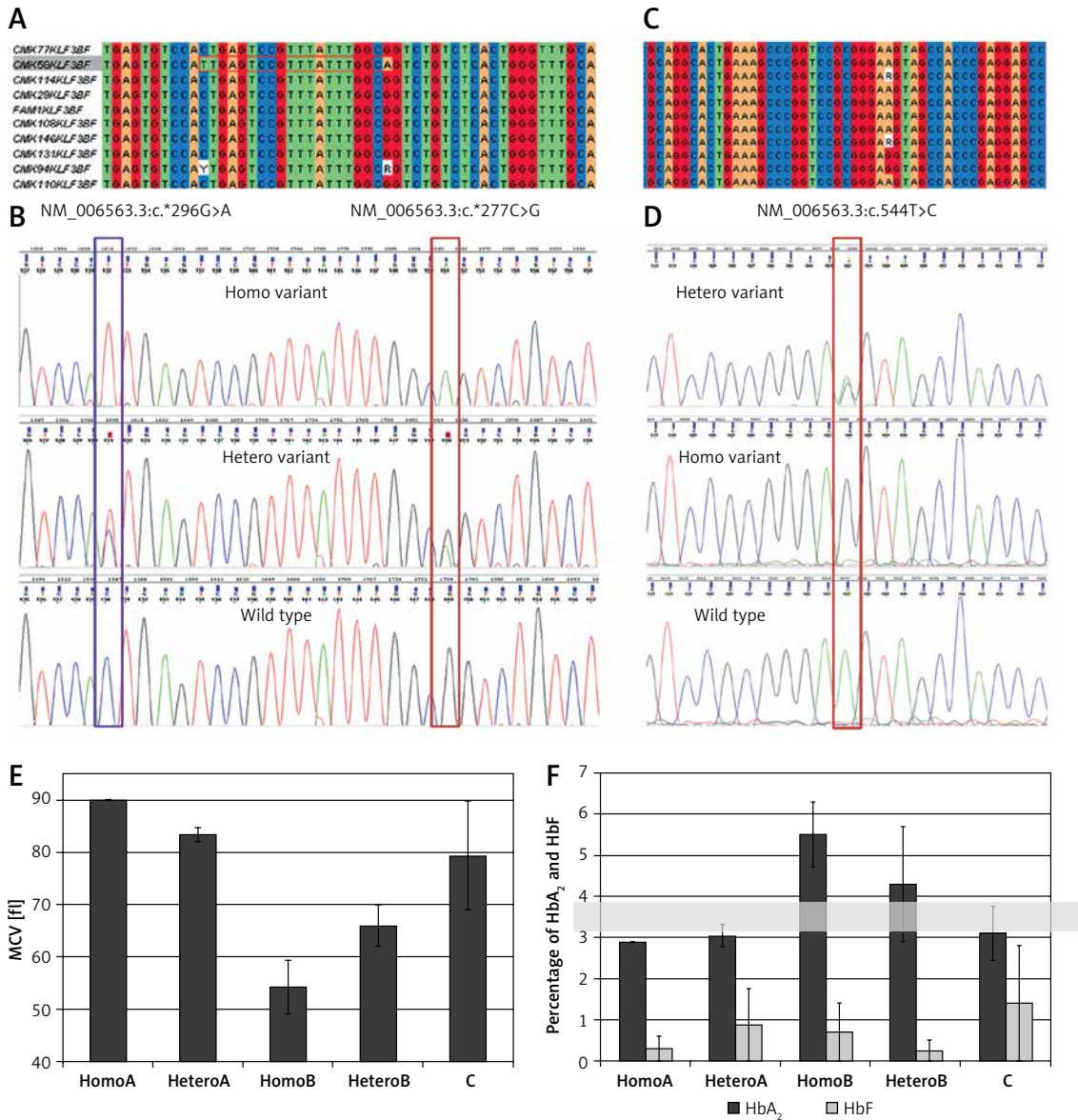

Figure 1. Sequencing chromatogram of newly identified variations in *KLF1* gene. **A** – Multiple sequence alignment of *KLF1* gene sequences, shows varied base pairs on 3′ untranslated region at *296th and at *277th on *KLF1* gene sequences. **B** – Sequencing chromatogram and single base substitution (substituted base is indicated by the colored box) at the *296th (violet box) and at the *277th (nut brown box) positions. Homo variant: Homozygous variant sequence due to substitution at *296th G>A and *277th C>G in both the chromosomes. Hetero variant: Heterozygous variant sequence due to substitution at *296th G>A and *277th C>G in single chromosome resulted in peaks overlapping. Wild type: Wild type sequence of the sense strand. **C** – Multiple sequence alignment of all sequences, shows variegated base pairs on intronic region at 544th position. **D** – Sequencing chromatogram and single base substitution (substituted base is indicated by the nut brown box) at 544th position. Homo variant: Homozygous variant sequence due to substitution at 544th T>C in both the chromosomes. Hetero variant: Heterozygous variant sequence due to substitution at 544th T>C in single chromosome resulted in peaks overlapping. Wild type: Wild type sequence of the sense strand. **E** – Concentration of MCV (fl) in different groups of subjects with *KLF1* gene variations. HomoA: Homozygous for NM_006563.3:c.*296G>A and NM_006563.3:c.*277C>G. HeteroA: Heterozygous for NM_006563.3:c.*296G>A and NM_006563.3:c.*277C>G. HomoB: Homozygous for NM_006563.3:c.544T>C. HeteroB: Heterozygous for NM_006563.3:c.544T>C. C: Control. **F** – Percentage of HbA$_2$ and HbF values in different groups of subjects with *KLF1* gene variations. HomoA: Homozygous for NM_006563.3:c.*296G>A and NM_006563.3:c.*277C>G. HeteroA: Heterozygous for NM_006563.3:c.*296G>A and NM_006563.3:c.*277C>G. HomoB: Homozygous for NM_006563.3:c.544T>C. HeteroB: Heterozygous for NM_006563.3:c.544T>C. C: Control

Statistical analysis

For statistical analysis between the hematological data and the presence of various mutations in the *KLF1* gene, Student's *t*-test was used using IPM SPSS statistics 19.

Results

The present study was carried out to identify the influence of variations in the *KLF1* gene in Saudis with borderline HbA$_2$. Four variations were identified, including two 3′ untranslated region or 3′UTR NM_006563.3:c.*296G>A and NM_006563.3:c.*277C>G, and 2 exonic NM_006563.3:c.304T>C and NM_006563.3:c.544T>C variations for the first time from the Saudi Arabian population (Figure 1). But none of these variations have shown significance over this borderline HbA$_2$ through significance analysis using Student's *t*-test between the group with and without *KLF1* gene variations.

The presence of the borderline HbA$_2$ level is not specific to a particular β°-thalassemia variation or β$^+$-thalassemia variations in the Saudi population (Table I). A normal level of HbA$_2$ (< 3) has been observed in healthy Saudis with heterozygous β°-thalassemia variation (*n* = 2, HBB:c.25_26delAA; *n* = 22, HBB:c.20A>T), furthermore, the *KLF1* gene was found to be variation free in these individuals (Table II). Deep analysis of these subjects revealed the presence of the *HBA12* gene in five subjects, which supports an earlier report [13]. KLF1 is a sequence-specific DNA binding factor, which is an essential transcription factor for α and β globin genes [11]. Reports from various populations have shown that there is an association between the variations in *KLF1* with borderline HbA$_2$ level in β-thalassemia carriers [7–11]. Therefore, the present study was conducted to establish the association of variations in the *KLF1* gene in Saudi subjects with borderline HbA$_2$ and also to shed some light on the possible effects of this variation in the *KLF1* gene on the level of HbA$_2$. Sequence analysis of the *KLF1* gene from the subjects with HbA$_2$ < 3; HbA$_2$ = 3.1 to 3.9 and HbA$_2$ > 4.3 identified four variations, which were observed in a few samples in all the groups. They were found to be not specific for any of the three groups (Table II). Two 3′ untranslated region or 3′UTR substitution variations were found at base pair 296 and at 277 (NM_006563.3:c.*296G>A and NM_006563.3:c.*277C>G respectively). Notably, none of the subjects with HbA$_2$ > 4.3 were identified with α globin gene variants (Table I). The

Table II. Gene variations in Saudi subjects enrolled in the study

Parameter	Group		
	HbA$_2$ < 3	HbA$_2$ 3.1–3.9	HbA$_2$ > 4.3
n	88 (24 F, 64 M)	72 (16 F, 56 M)	52 (14 F, 38 M)
Subject with β°-thalassemia variation	24	60	52
Subject without β°-thalassemia variation	64	12	0
Subject without β°-thalassemia variation and with α globin genotype	$-\alpha_2^{3.7}/\alpha_1^{polyA-1}\alpha_2$ (*n* = 1) $\alpha_1\alpha_2/\alpha_1\alpha_{12}$ (*n* = 2) $-\alpha_2^{3.7}/\alpha_1\alpha_2$ (*n* = 13)	$\alpha_1\alpha_2/\alpha_1\alpha_{12}$ (*n* = 5) $-\alpha_2^{3.7}/\alpha_1\alpha_2$ (*n* = 7)	–
Subject with β°-thalassemia variation and with α globin genotype	$\alpha_1\alpha_2/\alpha_1\alpha_{12}$ (*n* = 2) $-\alpha_2^{3.7}/\alpha_1\alpha_2$ (*n* = 4) $\alpha_1\text{-}^{4.2}/\alpha_1\alpha_{12}$ (*n* = 2) $-\alpha_{12}^{3.7}/\alpha_1\alpha_{12}$ (*n* = 2)	$\alpha_1\alpha_2/\alpha_1\alpha_{12}$ (*n* = 5)$^{\#\$}$ $\alpha_1\alpha_{12}/\alpha_1\alpha_{12}$ (*n* = 2) $-\alpha_2^{3.7}/\alpha_1\alpha_2$ (*n* = 33)	$-\alpha_2^{3.7}/\alpha_1\alpha_2$ (*n* = 10)
β°-thalassemia variation	c.20A>T (*n* = 22) c.25_26delAA (*n* = 2)	c.20A>T (*n* = 56) IVS I-5 (G→C) (*n* = 2)$^\$$ c.9T>C (*n* = 1) c.17_18delCT (*n* = 2)$^\#$ c.9T>C (*n* = 1)	c.2T>C c.46delT NG_000007.3: g.71609_72227del619 c.20A>T; c.46delT c.93-23_94del c.25_26delAA
Other β variants or SNP	c.315+16G>C c.315+74T>G	c.315+16G>C c.315+74T>G	c.315+16G>C c.315+74T>G
KLF1 variants	c.544T>C; c.*296G>A c.*277C>G; c.304T>C	c.544T>C; c.*296G>A c.*277C>G; c.304T>C	c.544T>C; c.*296G>A c.*277C>G c.304T>C

$^\#$ and $^\$$ indicates those variations identified in the same sample, SNP – single nucleotide polymorphism.

variation NM_006563.3:c.304T>C was identified in many Saudis (n = 58 hetero; n = 18 homo) subjects with varied levels of HbA_2; hence we could say that this variation is also not specific to borderline HbA_2. The detailed study on the α globin genotype revealed that the prevalence of $-\alpha_2^{3.7}/\alpha_1\alpha_2$ is the most frequent (29.06%) among healthy Saudis (Table I). However, the α globin genotype, $-\alpha_2^{3.7}/\alpha_1\alpha_2$ is found to be the most frequent (55.55%) among healthy Saudis with borderline HbA_2 compared with the other groups ($HbA_2 < 3 = 20.45\%$; $HbA_2 > 4.3 = 13.51\%$).

Effects of the amino acid changes S102P and F182L due to the exonic variations such as NM_006563.3:c.304T>C and NM_006563.3:c.544T>C respectively on the KLF1 protein (PDB ID: 2I3B) were studied by structural modeling using earlier methodology using SWISS MODEL and PROCHECK [18–21]. Three-dimensional (3D) homology modeling of KLF1 protein from the wild and
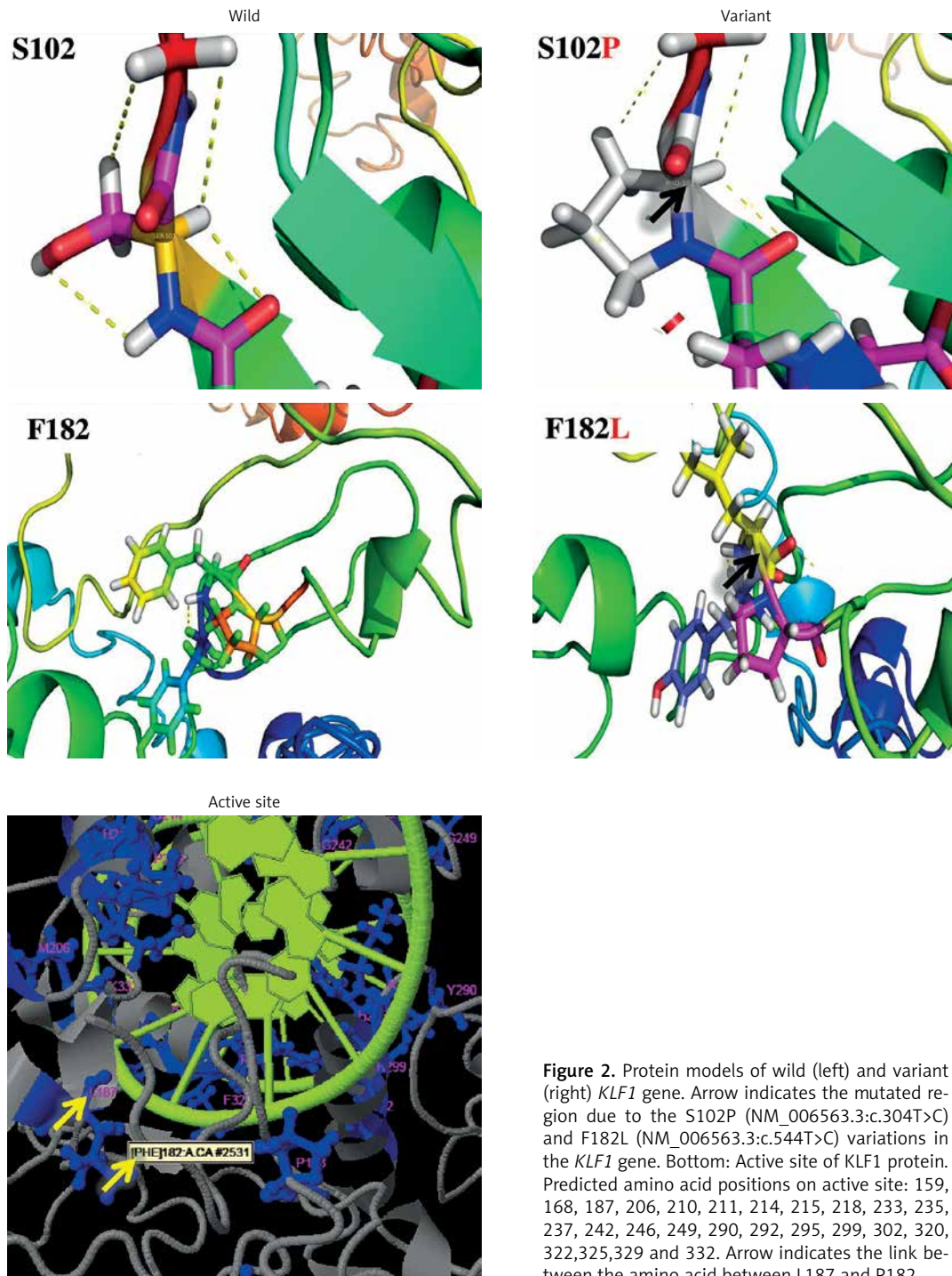

Figure 2. Protein models of wild (left) and variant (right) *KLF1* gene. Arrow indicates the mutated region due to the S102P (NM_006563.3:c.304T>C) and F182L (NM_006563.3:c.544T>C) variations in the *KLF1* gene. Bottom: Active site of KLF1 protein. Predicted amino acid positions on active site: 159, 168, 187, 206, 210, 211, 214, 215, 218, 233, 235, 237, 242, 246, 249, 290, 292, 295, 299, 302, 320, 322, 325, 329 and 332. Arrow indicates the link between the amino acid between L187 and P182

variants revealed that the variants S102P (Å 3.58) and F182L (Å 3.42) show significantly deviated RMSD (Å) compared to the wild type (Å 0) (Figure 2). The total energy values of S102P (2723945 kJ/mol) and F182L (1871 kJ/mol) variegated proteins were deviated from the wild (656 kJ/mol) protein. The interaction between the amino acid (L187) at the active site and studied amino acid (F182) as well as the changes in the RMSD (Å) due to the variation (F182L) show its biological significance (Figure 2). However, we could not identify any notable impact on the phenomes of the subjects with F182L.

Discussion

The allelic association or genotypic association of the NM_006563.3:c.304T>C with the levels of the HbA$_2$ was found to be insignificant (p = 0.9842; odd ratio 1.02 (95% CI: 0.46–2.2). However, studies from Italian and Chinese populations on the level of HbA$_2$ and the influence of variation in *KLF1* (K332Q; S270X; p.Ala298Pro, p.Thr334Arg; c.913+1G>A; p.His299Asp; p.Cys341Tyr; p.Glu5Lys) showed prominent changes [22–25]. These variants (K332Q; S270X; p.Ala-298Pro, p.Thr334Arg; c.913+1G>A; p.His299Asp; p.Cys341Tyr; p.Glu5Lys) do not exist in the Saudi population; therefore, these variants need not be included in screening.

Detailed analysis of the molecular nature of the α globin (*HBA1* and *HBA2*), β globin (*HBB*) and *KLF1* genes on Saudis with various levels of HbA$_2$ revealed that the *KLF1* gene or its variants was found to be not associated with the presence of borderline HbA$_2$. The rate of consanguinity among Saudis is high [26]. Hence, precise identification of individuals with β globin gene variation is necessary for distinguishing couples at risk for having progeny with β-thalassemia major of sickle cell disease, especially considering the level of HbA$_2$ for the identification of β-thalassemia carriers. Hence the identification of the $-\alpha_2^{3.7}/\alpha_1\alpha_2$ and the *HBA12* gene on the subjects with borderline HbA$_2$ should be incorporated in the existing premarital screening in Saudi Arabia to eliminate the erroneous diagnosis of β-thalassemia carriers as normal [27]. Enzymes may play a critical role; hence enzyme activities among these subjects can also be considered in future [28]. There is no evidence of the *HBD* gene variations in the subjects with borderline HbA$_2$ in Saudis. Hence, the δ globin gene and its association with the changes in the level of the HbA$_2$ should be studied in detail in Saudi subjects.

The occurrence of hemoglobin disorders is common among Saudis [1–5].

In conclusion, two exonic (NM_006563.3:c. 304T>C and NM_006563.3:c.544T>C) and two

3′ untranslated region or 3′UTR (NM_006563. 3:c.*296G>A and NM_006563.3:c.*277C>G) variations have been identified for the first time from an Arab population. None of these four variations are significantly associated with the Saudis with borderline HbA$_2$. α Globin genotype, $-\alpha_2^{3.7}/\alpha_1\alpha_2$ and the *HBA12* gene might be associated with borderline HbA$_2$ in Saudis. The real reason for the borderline HbA$_2$ in 44.5% of Saudis is still unclear.

Conflict of interest

The authors declare no conflict of interest.

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