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In silico prediction of *HBD* gene variants in the Iranian population

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Abstract

Background: The quantification of hemoglobin A_2 (Hb A_2; α2δ2) is used as a valuable test to differentiate α- and ß-thal carriers in clinical laboratories. Therefore, the *HBD* (δ-globin) gene variants could result in reduced levels of Hb A_2 and have implications for thalassemia screening programs. The aim of the present study was to predict the consequences of *HBD* gene variants identified in the Iranome project.

Results: The highest number of variants was in the Persian Gulf Islanders. The variants of p.Gln132Glu (*HBD*: c.394C>G), p.Gly17Arg (*HBD*: c.49G>C), p.Thr5Ile (*HBD*: c.14C>T), and p.Ala28Ser (*HBD*: c.82G>T) presented damage results in three or more prediction tools. In addition, it seems that the p.Gly30= (*HBD*: c.90C>T) decreases the use of authentic splice and, instead, creates a new donor splice site (DSS) or leads to the use of a cryptic DSS.

Conclusions: Most of these variants have been associated with a decrease in Hb A_2 levels. Due to the high mutational diversity in the *HBB* gene in the Iranian population and the use of Hb A_2 quantification to differentiate α- and ß-thal carriers among Iranian clinical laboratories, some attention should be taken to a possible co-inheritance of *HBD* gene variants to avoid the misdiagnosis of ß-thal carriers.

Keywords: Hemoglobin A_2, δ-globin gene, Variant, Thalassemia, Iran

Background

Hemoglobin A (Hb A; α2β2, 96–98%), hemoglobin A_2 (Hb A_2; α2δ2, less than 3%), and hemoglobin F (Hb F; α2γ2, less than 1%) are three main components of the total hemoglobin observed in normal adults. Alpha and beta thalassemia (α- and ß-thal) are due to mutations in the *HBA1*/*HBA2* and *HBB* genes, respectively [1–3].

As a member of the ß-globin gene family, the *HBD* or δ-globin gene is located on chromosome 11. This gene is positioned on the 5′ side of the *HBB* gene and encodes a 147-amino acid protein that differs from ß-globin in only 10 amino acids [3]. The HbVar database (http://globin.bx.psu.edu/hbvar/) is known as a database of information about Hb variants and mutations that cause thalassemia. Although more than 120 variants have been identified in the *HBD* gene so far, they are far fewer in number than the number of variants reported in the *HBB* gene [4]. This is because the *HBD* gene variants are clinically "silent". Most of the *HBD* gene variants are missense and result in reduced levels of Hb A_2 [5].

On the other hand, Hb A_2 accounts for only a small fraction of total hemoglobin and has no known physiological role [3]. However, Hb A_2 quantification is used as a valuable test to differentiate α- and ß-thal carriers in clinical laboratories; its level is normal or slightly reduced in α-thal carriers and is increased to more than 4% in ß-thal carriers. Therefore, in the populations such as Iran in which ß-thal is a serious problem in the health system [6–8], any factors affecting the level of Hb A_2 could have implications for thalassemia screening programs.

The Iranome database (http://www.iranome.ir/) has recorded the genomic variants found in 800 healthy individuals from eight major ethnic groups in Iran, including Arabs, Azeris, Balochs, Kurds, Lurs, Persians, Persian Gulf Islanders, and Turkmen, with 100 individuals per ethnic group [9]. In this study, we used 14 in silico prediction

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tools to identify the deleterious possibility of the *HBD* variants reported in the Iranome database.

Methods

All studies related to the identification of *HBD* gene variants in recent years in the Iranian population as well as all *HBD* gene variants identified in the Iranome project were extracted. To screen the rare variants with frequencies residing around or under 1%, the allele frequency for each variant discovered in 1000 Genomes Project and Genome Aggregation Database (gnomAD) (Available at http://grch37.ensembl.org/Homo_sapiens/Info/Index) was used as the reference. In addition, variants were checked for previously reported in the Single Nucleotide Polymorphism database (dbSNP) (https://www.ncbi.nlm.nih.gov/snp/), ITHANET web portal (https://www.ithanet.eu/), HbVar database, as well as in the literature. The ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) was used to search for known variants along with their clinical significances.

Pathogenicity of *HBD* variants was predicted by using in silico tools such as MutationTaster (http://www.mutationtaster.org/), SIFT (https://sift.bii.a-star.edu.sg/), PROVEAN (http://provean.jcvi.org/index.php), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), FATHMM-XF (http://fathmm.biocompute.org.uk/fathmm-xf/), *I-Mutant disease (*http://gpcr.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi*)*, SNPs&GO (https://snps.biofold.org/snps-and-go/), PhD-SNP^{g} (https://snps.biofold.org/phd-snpg/index.html), PMut (http://mmb.irbbarcelona.org/PMut) and CADD (https://cadd.gs.washington.edu/).

Deleterious thresholds used in the above tools were as follows: SIFT < 0.05 [10], PolyPhen-2 > 0.5 [11], PROVEAN < − 2.5 [12], FATHMM > 0.5 [13], SNPs&GO > 0.5 [14], PhD-SNP^{g} > 0.5 [15], and PMut > 0.5 [16]. For CADD, we used the highest phred-like score cutoff recommended by the authors, i.e., 20 [17]. MutationTaster predicts an alteration as one of four possible types: disease causing (i.e., probably deleterious), disease-causing automatic (i.e., known to be deleterious), polymorphism (i.e., probably harmless), and polymorphism automatic (i.e., known to be harmless). In addition, we used VarSEAK (https://varseak.bio/), MaxEntScan (http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/), and NNSplice (https://www.fruitfly.org/seq_tools/splice.html), as some splice site prediction tools to predict the effect of variants on splicing events. The output of these tools are based on score (%), maximum entropy score, confidence score (0-1), and score (0-1), respectively. Higher score implies a higher probability/confidence of the sequence being a true splice site [18].

Here, we used VarSome database (http://varsome.com) for the interpretation of sequence variants [19]. This database uses ACMG standards and guidelines for interpretation [20]. The gene reference sequence was NG_063112.2. The NM_000519.4 was used to determine the variant position. Position of the variants in protein was determined based on UniProtKB/SwissProt P02042.

Results

Among the 800 healthy individuals studied in the Iranome project, *HBD* gene variants have been reported in 46 individuals from different ethnicities. Accordingly, the highest number of variants was in the Persian Gulf Islanders, followed by Balochs, Lurs, Kurds, Persians, Turkmen, Azeris, and Arabs, respectively (Table 1). According to NM_000519.4 reference transcript, a total of 16 different single nucleotide variations (SNVs), including seven exonic and nine intronic variants, were identified in the *HBD* gene. All variants were single nucleotide substitutions, and no insertion or deletion variants were observed. In addition, all variants were detected in heterozygous states (Table 1).

Except for *HBD*: c.315+55G>T and *HBD*: c.92+43A>G, the other intronic variants were previously recorded in dbSNP. However, none of the intronic variants were reported in the ITHANET, HbVar, and ClinVar databases (Table 1). Also, analysis on the MutationTaster, *FATHMM-XF,* PhD-SNP^{g} (except for *HBD:* c.315+199A>G), and CADD tools showed that all intronic variants were in the category of benign/polymorphisms. In addition, based on the VarSEAK, MaxEntScan, NetGene2, and NNSplice tools, none of these variants had effect on splicing events (Table 2).

Exonic variants were divided into two groups, synonymous SNVs and nonsynonymous SNVs, which accounted for two and five variants, respectively (Fig. 1). The nonsynonymous variants were p.Gln132Glu (*HBD*: c.394C>G), p.Asp53Glu (*HBD*: c.159 T>G), p.Gly17Arg (*HBD*: c.49G>C), p.Thr5Ile (*HBD*: c.14C>T), and p.Ala28Ser (*HBD*: c.82G>T). Although p.Asp53Glu (*HBD*: c.159 T>G) showed neutral results in all 14 tools, the other four nonsynonymous SNVs presented damage results in three or more prediction methods (Table 3). The analysis of *HBD*: c.82G>T variant on the splice site prediction tools revealed that the replacement of guanine with thymine at position c.82 activates a cryptic donor splice site (DSS) at c.78. This new splice site was much stronger than the authentic splice site at c.92+1 position (Fig. 2a).

None of the two synonymous SNVs, p.Gly30= (*HBD*: c.90C>T) and p.His98= (*HBD*: c.294C>T), were found in both ITHANET and HbVar databases (Table 1). Unlike the *HBD*: c.294C>T variant, *HBD*: c.90C>T had deleterious results on MutationTaster, CADD, and PhD-SNP^{g} web tools (Table 3). In addition, based on the splice site prediction tools, the replacement of cytosine with thymine at position 90 decreases the score for the use of

Table 1 Variants observed in the *HBD* gene in the Iranian population, based on Iranome project (http://www.iranome.ir/)

Variant genomic position/rs ID	Transcript/protein consequence	ClinVar	Ethnicity	Reported in ITHANET	Reported in HbVar	Exon/ intron	Allele frequency (%)
11:5254244 G/C	c.394C>G/p.Gln132Glu	NR	Kurd	Yes/causative	Yes	Exon 3	1/1600 (0.06)
11:5254398 A/G (rs200027473)	c.316-76 T>C	NR	Turkmen	No	No	Intron 2	1/1600 (0.06)
11:5255166 C/A (rs1589897475)	c.315+55G>T	NR	Azeri	No	No	Intron 2	1/1600 (0.06)
11:5255377 A/C (rs757106601)	c.159 T>G/p.Asp53Glu	NR	Arab	No	No	Exon 2	1/1600 (0.06)
11:5255477 G/A (rs149402829)	c.93-34C>T	NR	Azeri	No	No	Intron 1	1/1600 (0.06)
11:5255529 T/C	c.92+43A>G	NR	Kurd	No	No	Intron 1	1/1600 (0.06)
11:5255574 G/A (rs1223305519)	c.90C>T/p.Gly30=	NR	Arab	No	No	Exon 1	1/1600 (0.06)
11:5255615 C/G (rs34012192)	c.49G>C/p.Gly17Arg	Other	Persian Gulf Islander	Yes/causative	Yes	Exon 1	1/1600 (0.06)
11:5254402 C/T (rs374210782)	c.316-80G>A	NR	Baloch	No	No	Intron 2	2/1598 (0.13)
11:5254424 T/C (rs73400693)	c.316-102A>G	NR	Baloch, Persian Gulf Islander	No	No	Intron 2	2/1590 (0.13)
11:5255022 T/C (rs181334077)	c.315+199A>G	NR	Baloch	No	No	Intron 2	1/716 (0.14)
11:5255075 A/C (rs77044643)	c.315+146T>G	NR	Baloch, Persian Gulf Islander	No	No	Intron 2	2/1118 (0.18)
11:5254354 A/G (rs73400692)	c.316-32T>C	NR	Baloch, Persian Gulf Islander, Turkmen	No	No	Intron 2	3/1600 (0.19)
11:5255650 G/A (rs35406175)	c.14C>T/p.Thr5Ile	Uncertain significance	Multiple	Yes/causative	No	Exon 1	6/1600 (0.38)
11:5255242 G/A (rs61746501)	c.294C>T/p.His98=	Benign	Multiple	No	No	Exon 2	9/1600 (0.56)
11:5255582 C/A (rs35152987)	c.82G>T/p.Ala28Ser	Conflicting interpretations	Multiple	Yes/causative	Yes	Exon 1	9/1600 (0.56)

Human genome reference: hg19/GRCh37; gene reference sequence: NG_063112.2; mRNA reference sequence: NM_000519.4; UniProtKB/Swiss-Prot: P02042.2
NR not reported, *NA* not available

authentic DSS at c.92+1 and, instead, creates a new DSS at c.89 position or leads to the use of a cryptic splice site located at 16 nt upstream of authentic DSS (Fig. 2b).

Discussion

More than 1.5 million variants have been identified in the genomes of individuals studied in the Iranome project [9]. Using 14 prediction tools, we evaluated a number of 16 *HBD* gene variants reported in the Iranome database (Table 1). Based on the ACMG guidelines, none of these variants were categorized as pathogenic or likely pathogenic (Tables 2 and 3).

Zhang et al. [21] reported p.Gln132Glu (*HBD*: c.394C>G) as a novel δ-globin variant in a healthy Chinese 35-year-old man in 2019 and named it Hb A2-Puer. The hematological and electrophoretic data related to this Hb variant in heterozygous state were as follows: Hb (g/dL) 16.1, MCV (fL) 85.2, MCH (pg) 29.0, Hb A (%) 97.4, Hb A_2 (%) 1.3, and Hb X (%) 1.4. Our analysis showed that *HBD*: c.394C>G is a variant with deleterious effects on MutationTaster, FATHMM-XF, I-Mutant disease, and PhD-SNP^g, and neutral/benign on SIFT, PROVEAN, PolyPhen-2, SNPs&GO, VarSEAK, and Pmut prediction tools. In addition, with a score of 18.06 in the CADD web tool, this variant could not get the phred-like score cutoff at 20 to locate in the top 1% probability of being deleterious. On the other hand, the same variant has been reported on the *HBB* gene (Hb Camden: *HBB*: c.394C>G) with conflicting interpretations of pathogenicity, from silent or likely benign/uncertain significance in the literature [22] or in the ClinVar database, respectively, to causative in the ITHANET database. Finally, based on the ACMG guidelines, the *HBD*: c.394C>G variant was classified as a variant of uncertain significance (VUS) [20]. According to the Iranome database, the *HBD*: c.394C>G variant has been observed in a Kurdish healthy individual in heterozygous form. Since the only report of this variant in the literature is related to Zhang et al.'s [21] study, it can be assumed that the present study is the second one to report this variant in the world and the first study to annotate it in Iran.

The *HBD*: c.49G>C variant, also known as Hb A2′ or Hb B2, is one of the most common δ-globin gene variants, mainly found in Black families and occurs in nearly 1% of African-Americans [1]. The same substitution was found on the *HBB* and *HBG2* genes [Hb D-Bushman (*HBB*: c.49G>C) and Hb F-Melbourne (*HBG2*: c.49G>C), respectively]. The Hb A2-Yialousa (*HBD*: c.82G>T) and *HBD*: c.14C>T had also been reported in the Iranome database. The Hb A2-Yialousa has been shown to be the

Table 2 In silico prediction of intronic variants observed in the *HBD* gene in the Iranian population, based on the Iranome project (http://www.iranome.ir/)

	c.316-76T>C	c.315+55G>T	c.93-34C>T	c.92+43A>G	c.316-80G>A	c.316-102A>G	c.315+199A>G	c.315+146T>G	c.316-32T>C
Mutation Taster	P	P	P	P	P	P	P	P	P
FATHMM-XF	Benign	Benign	Benign	Benign	Benign	Benign	Benign	Benign	Benign
CADD	8.948	0.201	2.825	5.735	5.194	1.516	3.008	3.105	6.307
VarSEAK	No splicing effect	No splicing effect	No splicing effect	No splicing effect	No splicing effect	No splicing effect	No splicing effect	No splicing effect	No splicing effect
MaxEntScan	No change	No change	No change	No change	No change	No change	No change	No change	No change
NetGene2	No change	No change	No change	No change	No change	No change	No change	No change	No change
NNSplice	No change	No change	No change	No change	No change	No change	No change	No change	No change
PhD-SNPg	Benign	Benign	Benign	Benign	Benign	Benign	Pathogenic	Benign	Benign
Frequency in 1000 genomes Project	0.001	NA	0.0002	NA	0.0002	0.014	0.002	0.06	0.019
Frequency in gnomAD	0.00006	NA	0.000008	NA	NA	0.013	0.003	0.055	0.018
ACMG	VUS	VUS	VUS	VUS	VUS	Likely benign	Likely benign	Benign	Benign

P polymorphism, *NA* not available, *VUS* variant of uncertain significance

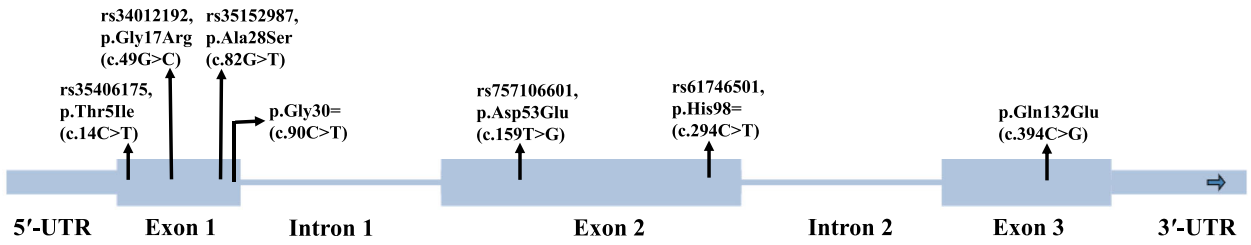

Fig. 1 Location of synonymous SNVs and nonsynonymous SNVs observed in the *HBD* gene in the Iranian population, based on the Iranome project (http://www.iranome.ir/)

most common *HBD* gene variant in the Mediterranean area [1] as well as in Iran [23], and a rare variant in China [24]. It seems that the replacement of guanine with thymine at position c.82 activates a cryptic DSS at the upstream (Fig. 2a). The *HBD*: c.14C>T variant was first identified in Greek Cypriots [25]. The frequency of this variant is low and has been reported in other populations such as Oman [26] and China [24]. All of these variants have been associated with a decrease in Hb A_2 levels [23–26]. Based on our analyses, these variants presented damage results in three or more prediction methods (Table 3). In addition, according to ACMG guidelines, they were classified as likely benign. Therefore, the pathogenicity or neutrality of them remains unknown.

Another missense variant, *HBD*: c.159 T>G, was previously recorded in the dbSNP (rs757106601). However, there was no information associated to this variant in the literature, as well as in the ClinVar, ITHANET, and HbVar databases (Table 1). In addition, the same variant has not been reported on the *HBB* gene. Our analysis showed neutral results related to this variant in all 14

Table 3 In silico prediction of exonic variants observed in the *HBD* gene in the Iranian population, based on the Iranome project (http://www.iranome.ir/)

Tool	c.394C>G/ p.Gln132Glu	c.159 T>G/ p.Asp53Glu	c.90C>T/ p.Gly30=	c.49G>C/ p.Gly17Arg	c.14C>T/ p.Thr5Ile	c.294C>T/ p.His98=	c.82G>T/ p.Ala28Ser
MutationTaster	Disease causing	Polymorphism	Disease causing	Polymorphism	Polymorphism	Disease causing	Disease causing
SIFT	Tolerated	Tolerated	Tolerated	Deleterious	Deleterious	Tolerated	Tolerated
PROVEAN	Neutral	Neutral	Neutral	Deleterious	Deleterious	Neutral	Neutral
PolyPhen-2	Benign	Benign	NA	Benign	Possibly damaging	NA	Possibly damaging
FATHMM-XF	Pathogenic	Benign	Benign	Benign	Benign	Benign	Benign
I-Mutant Disease	Disease	Neutral	NA	Disease	Neutral	NA	Neutral
SNPs&GO	Neutral	Neutral	NA	Disease	Neutral	NA	Neutral
CADD	18.06	11.91	23.5	0.904	15.64	7.897	23
VarSEAK	No splicing effect	No splicing effect	New DSS	No splicing effect	No splicing effect	No splicing effect	Cryptic DSS activated
MaxEntScan	No change	No change	New DSS	No change	No change	No change	Cryptic DSS activated
NetGene2	No change	No change	New DSS	No change	No change	No change	Cryptic DSS activated
NNSplice	No change	No change	New DSS	No change	No change	No change	Cryptic DSS activated
PhD-SNPg	Pathogenic	Benign	Pathogenic	Benign	Benign	Benign	Pathogenic
Pmut	Neutral	Neutral	NA	Neutral	Neutral	NA	Neutral
Frequency in 1000 Genomes Project	NA	NA	NA	0.003	0.001	0.008	0.001
Frequency in gnomAD	NA	0.000004	NA	0.003	0.001	0.008	0.001
ACMG	VUS	Likely benign	VUS	Likely benign	Likely benign	Likely benign	Likely benign

NA not available, *DSS* donor splice site, *VUS* variant of uncertain significance

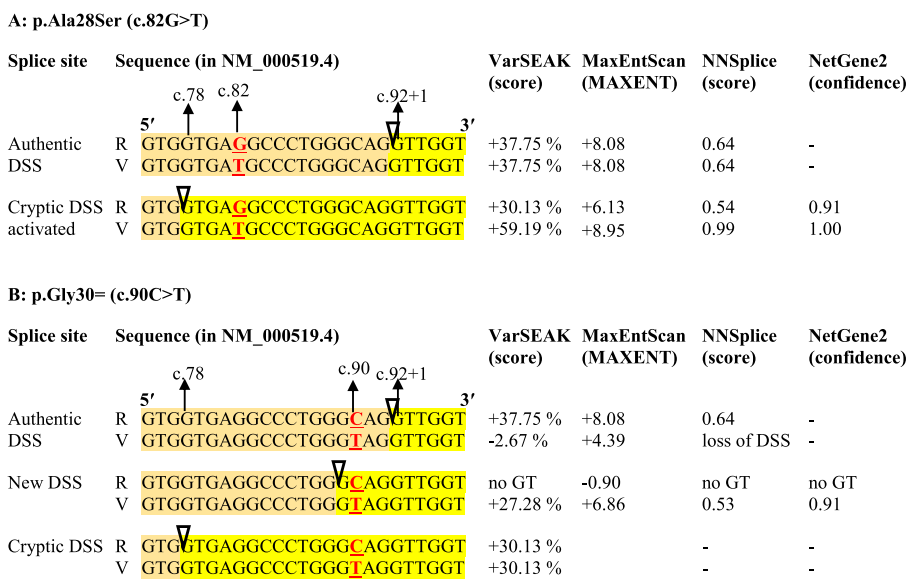

Fig. 2 The predicted splicing effects of p.Ala28Ser (*HBD*: c.82G>T) (**a**) and p.Gly30 = (*HBD*: c.90C>T) (**b**) variants. The replacement of guanine with thymine at position c.82 activates a cryptic donor splice site (DSS) at c.78. This new splice site is much stronger than the authentic splice site at c.92+1 position (**a**). The replacement of cytosine with thymine at position c.90 decreases the score for the use of authentic DSS at c.92+1 and instead, creates a new DSS at c.89 position or leads to the use of a cryptic splice site located at c.78 (**b**). Gold color means exon sequence; yellow color means intron sequence; triangle means splice site

prediction tools used in the present study and classified as VUS (Table 3).

Two synonymous *HBD* gene variants had been reported in the Iranome database (Tables 1 and 3). The p.Gly30= (*HBD*: c.90C>T) variant had deleterious results on MutationTaster, CADD, and PhD-SNP^{g} web tools (Table 3). It seems that the replacement of cytosine with thymine at position 90 decreases the score for the use of authentic DSS and, instead, creates a new DSS or leads to the use of a cryptic DSS (Fig. 2b). The same variant was found on the *HBB* gene, p.Gly30= (*HBB*: c.90C>T), which has been categorized as a pathogenic variant in the ClinVar database. According to the ACMG guidelines, *HBD*: c.90C>T was classified as VUS (Table 3). This variant was reported for the first time in the Iranome database in a healthy individual with Arab ethnicity. To the best of our knowledge, there is no report of this variant in the literature so far.

Thalassemia is a serious health problem in the Iranian population. Numerous studies conducted in Iran have shown a high mutational diversity for α- and ß-thal diseases [27–32]. However, to our knowledge, only a limited number of studies have been performed in Iran to identify the *HBD* gene variants. In fact, with the exception of the study by Kordafshari et al. [23], which reported the spectrum of *HBD* gene variants in 21 individuals, other studies were case reports that identified a specific variant in one or a limited number of individuals [33–35]. Therefore, at least six different types of variants in the *HBD* gene were reported before in the Iranian population: Hb A2-Yialousa (*HBD*: c.82G>T), Hb A2-Coburg (*HBD*: c.350G>A), Hb A2-NYU (*HBD*: c.39 T>A), Hb A2-Etolia (*HBD*: c.257 T>C), Hb A2-Fitzroy (*HBD*: c.428C>A), and *HBD*: c.92+5G>T. The *HBD*: c.82G>T and *HBD*: c.92+5G>T variants were the most frequent ones among Iranian population [23, 33–35].

Conclusions

Given the small number of studies performed on the *HBD* gene in Iran and the fact that the *HBD* gene variants are clinically "silent," it can be assumed that the spectrum of variants of this gene in our population is much wider. Evidence of this claim is revealed by the *HBD* gene variants identified in individuals who participated in the Iranome project. Out of the 16 *HBD* gene variants reported in the Iranome database, five variants including *HBD*: c.394C>G, *HBD*: c.49G>C, *HBD*: c.82G>T, *HBD*: c.14C>T, and *HBD*: c.90C>T showed the potential of deleterious effects in the present study. All of these variants, except for *HBD*: c.90C>T as a novel variant, have been associated with a decrease in Hb A_2 levels. Due to the high mutational diversity in the *HBB* gene in the Iranian population and the use of Hb A_2 quantification to differentiate α- and ß-thal carriers among Iranian clinical laboratories, some attention should be taken to a possible co-inheritance of *HBD* gene variants to avoid the misdiagnosis of β-thal carriers.

Abbreviations

Hb A: Hemoglobin A; Hb A_2: Hemoglobin A_2; HB F: Hemoglobin F; α-thal: Alpha thalassemia; ß-thal: Beta thalassemia; SIFT: Sorting Intolerant From Tolerant; PROVEAN: Protein Variation Effect Analyzer; Polyphen-2: Polymorphism Phenotyping v2; FATHMM-XF: Functional Analysis Through Hidden Markov Models–Extra Features; PhD-SNP^g: Predicting human Deleterious SNPs in human genome; CADD: Combined Annotation Dependent Depletion; SNV: Single nucleotide variation; ACMG: American College of Medical Genetics and Genomics; NNSplice: Splice Site Prediction by Neural Network; gnomAD: Genome Aggregation Database

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Authors' contributions

KM designed the study, monitored data collection for the whole research, analyzed and interpreted data, and drafted and revised the manuscript. AM collected data and drafted the manuscript. MK collected data. All authors have read and approved the manuscript.

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Availability of data and materials

The datasets analyzed during the current study are available in the Iranome database (http://www.iranome.ir/).

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran (Ethics code: IR.KUMS.REC.1399.797, project number: 990719).
In this study, we have only used the Iranome project data, and the consent form was previously obtained from all participants by Iranome project implementers.

Consent for publication

Not applicable.

Competing interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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