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Does the c.-273T>C variant in the upstream region of the *HBB* gene cause a thalassemia phenotype?

TO THE EDITOR: Beta thalassemia is a hereditary disease that results from mutations in the *HBB* gene, leading to genetic defects in the production of beta-globin chains [1, 2]. *HBB* encodes beta-globin, a subunit of hemoglobin. In adults, hemoglobin is normally made up of four protein subunits, including two subunits of beta-globin and two subunits of alpha-globin, with the latter produced from *HBA*. Mutations in *HBB* can result in either beta-plus (B^+) thalassemia that is responsible for a less severe form of thalassemia (caused by a decrease in beta-globin production) or beta-zero (B^0) thalassemia that is the severe type of the disease (caused by a total lack of beta-globin) [3-8].

The mutations usually include missense or nonsense types, but other types, such as deletions of the beta-globin gene and surrounding regions, also have been identified in thalassemia patients. According to the Human Gene Mutation Database (HGMD), currently, 835 disease-causing mutations have been found in *HBB*, including 404 missense/nonsense, 118 small deletions, 97 gross deletions, 73 regulatory, 53 splicing, 44 small insertions, 21 complex rearrangements, 19 small indels, and six gross insertions (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=*HBB*).

In addition, different studies have reported variants with unknown significance in the 5′ region, near the splice sites, and in the 3′ area of *HBB*, including c.-273T>C (upstream of the gene) [9]; until date, there have been no comprehensive data regarding its role in the phenotype of thalassemia. The goal of this study was to clarify the significance of this variant using segregation and bioinformatics analysis. Thus, from our large number of samples recruited from cases of minor thalassemia, we collected data regarding their laboratories and genetic studies. All patients provided informed consent before undergoing molecular testing for *HBB* and *HBA* mutation analysis. This study was approved by the institutional review board of the Comprehensive Medical Genetics Center, Shiraz University of Medical Sciences (Approval No. 95.113.). Genomic DNA was extracted from the peripheral blood lymphocytes of these samples using DNA Extraction Kits (Yekta Tajhiz, Iran) according to the manufacturer's instructions, and the DNA concentration was measured by NanoDrop (ND1000, USA) and stored at -20°C until use.

Sequences covering all coding and important non-coding regions of *HBB* and *HBA 1* and *2* genes were amplified by PCR. The total volume used for the PCR was 50 μL including 1 μL of each primer (20 pmol/μL), 2 μL DNA template (50–200 ng), 25 μL TEMPase Hot Start 2x Master Mix Blue (Ampicon, A290806), and 21 μL dH2O. The PCR reactions were carried out according to Amplicon TEMPase Hot Start protocol and programs. Ten microliters of the PCR products were visualized on 2% agarose gel containing SYBR Safe. For mutation analysis for *HBA 1* and *2* genes, ViennaLab StripAssays was used to locate all important deletions that were not detected by standard PCR.

From 200 samples of different types of suspected minor thalassemia, we found 12 cases with c.-273T>C. It is worth noting that one of the cases had this variant in a homozygous pattern and another of the cases with c.-273T>C had a pathogenic mutation. All heterozygous cases for c.-273T>C (cases 1–10) and also that of the homozygous form, including

Table 1. Laboratory findings and identified variants in *HBB* and *HBA1*.

Case No.	Gender	Hb (g/dL) F: 12.0–16.0 M: 13.5–17.5	HbA1 (%) 95–98	HbA2 (%) 1.5–3.0	MCV (fL) 80–100	MCH (pg/cell) 26–34	*HBA1* and *HBA2* Variants	*HBB* c.-273T>C	*HBB* Pathogenic mutation
1	F	13.3	97.4	2.1	76.6	23.9	Normal	Het	-
2	F	13.9	97	2.5	78.92	25.62	Het $a^{3.7}$ deletion	Het	-
3	M	15.8	97.3	2.7	77.1	25.5	Het α2 Poly A-2[a)]	Het	-
4	M	13.2	97.79	2.21	70	21	Het α2 Poly A-2 and Het $a^{3.7}$ deletion	Het	-
5	F	13.2	97.4	2.6	73.4	23.7	Het α2 cd19	Het	-
6	M	15.1	97.7	2.3	71.9	22.9	Het α2 Poly A-2	Het	-
7	F	13.8	96.68	2.4	75.0	24.3	Het α2 Poly A-2	Het	-
8	M	13.8	96.9	2.5	70.5	22.4	[e] Homo $a^{3.7}$ deletion	Het	-
9	M	14.3	97.5	2.5	75.92	24.62	Het $a^{3.7}$ deletion	Het	-
10	F	10.2	97.6	2.4	69.3	20.7	c.22A>T in *HBA2*	Het	-
11	M	14.6	96.0	4.0	70.2	21.9	Normal	Het	Het c.92+6T>C
12	F	13.3	97.4	2.6	74.5	25.3	Het α2 IVS1 -5 bp deletion	Hom	-

[a)]α2 Poly A-2: AATAAA>AATGAA
Abbreviations: F, female; Het, Heterozygous; Hom, Homozygous; M, male.

case 12 without any pathogenic mutations in *HBB*, had *HBA*2 levels in the normal range (Table 1). However, the case with c.-273T>C (case 11) that also had a pathogenic c.92+6T>C mutation showed an HBA2 level of 4.0%, indicating that c.-273T>C has no effect on HBB protein. In *HBA 1* and *2*, we found the different mutations listed in Table 1, which shows why some cases without any mutations in *HBB* had positive laboratory findings.

We also used bioinformatics software, such as FATHMM, CADD, and PhastCons programs, to predict the effects of this variant in the non-coding region of *HBB*. The FATHMM program (http://fathmm.biocompute.org.uk/disease.html) uses information concerning sequence homology and its score ranges between -16.13 and 10.64. If a score is lower than -1.5, then the corresponding nonsynonymous SNP (NS) is predicted as DAMAGING. The non-coding score of FATHMM for this variant was 0.042, which predicts that this variant does not have functional effects on the HBB protein. For CADD program, mutations with scores ≥10 are predicted to be the 10% most deleterious substitutions, whereas scores ≥20 signify the 1% most deleterious effects. Using SeattleSeq Annotation (http://snp.gs.washington.edu/SeattleSeqAnnotation138/), we found that the CADD score for this variant was 0.146, which predicts that c.-273T>C is a benign variant.

The phastCons program was also used to identify conserved genomic regions. Higher scores indicate a higher probability that the base is in a conserved element. The PhastCons score of the c.-273T>C variant was 0.250, suggesting that the region of the variant is less conserved among vertebrates.

In conclusion, our data confirmed that c.-273T>C does not impose any effect on the function of *HBB* protein and should be considered as a benign variant.

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Comparison of the acute erythropoietic capacities of erythropoietin and U-74389G in terms of hemoglobin levels

TO THE EDITOR: This study compared the erythropoietic capacities of erythropoietin (EPO) and antioxidant drug U-74389G based on the findings of 2 preliminary studies. Hemoglobin augmentation was evaluated using a hypoxia reoxygenation (HR) protocol in an animal model. Hemoglobin levels were evaluated at the 60th reoxygenation minute (for groups A, C, and E) and at the 120th reoxygenation minute (for groups B, D, and F) in 60 rats. Groups A and B were administered no drugs, groups C and D were administered EPO, and groups E and F were administered U-74389G. The first preliminary study of EPO did not show a significant increase in hemoglobin levels. However, the second preliminary study of U-74389G showed a significant increase in hemoglobin levels by 2.5±1.3% (P=0.0423). These 2 studies were co-evaluated because they were conducted in the same experimental setting. In non-deficient EPO rats, U-74389G demonstrated an approximately 2-times higher erythropoietic potency than EPO (P=0.0000). This is because the anti-oxidant capacity of U-74389G increased the acute erythropoietic potency.

A previous study claims that U-74389G harbors a remarkable acute erythropoietic capacity [1]. U-74389G is a novel antioxidant factor and has shown tissue protective effects in tissue hypoxia and reoxygenation (HR) experiments. U-74389G, also known as 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-pregna-1,4,9(11)-triene-3,20-dione maleate salt, prevents both arachidonic acid-induced and iron-dependent lipid peroxidation. It has been shown to protect against HR injury in animal heart, liver, and kidney models. These membrane-associating antioxidants are particularly effective in preventing permeability changes in brain microvascular endothelial cells monolayers. Lazaroids, or 21-aminosteroids, a novel series of glucocorticoid compounds, scavenge free radicals. U-74389G is one of the 132 similar lazaroid compounds. It has a molecular weight of 726.90406 g/mol and demonstrates selective action on the vascular endothelium with vitamin E-like properties.

However, the erythropoietic capacity of U-74389G appears more comprehensible when compared with that of a standard known drug. One such well-studied drug, wherein erythropoietic capacity was confirmed (P=0.3984), is EPO. Indeed, EPO has been implicated in over 29,946 known biomedical studies. Among these studies, 30.65% concern tissue HR experiments. However, only a few reports that were found to be related with this study did not completely address the specific matter of antioxidant factors. The aim of this experimental work was to compare the acute erythropoietic capacities of U-74389G and EPO in a non-deficient EPO rat model using an HR protocol. Their effects were assessed on the basis of increase in hemoglobin levels.

The veterinarian licenses for the research were provided under the 3693/12-11-2010 & 14/10-1-2012 decisions. The institute and place of experiment are mentioned in the related references [1, 2]. The experimental protocol, which involved Albino female Wistar rats, adhered to the ethical rules of the relevant organization. For 7 days pre-experimentally, the rats were placed under normal housing and fed *ad libitum* in the laboratory. Continuous intra-experimental general anesthesia, oxygen supply, electrocardiography, acidometry, and post-experimental euthanasia were provided. Subsequently, 16–18-week-old rats were randomly divided into 6 groups (N=10) according to the HR protocol: hypoxia for 45 minutes followed by reoxygenation for 60 minutes (group A); hypoxia for 45 minutes followed by reoxygenation for 120 minutes (group B); hypoxia for 45 minutes followed by immediate intravenous (IV) EPO administration and reoxygenation for 60 minutes (group C); hypoxia for 45 minutes followed by immediate IV EPO administration and reoxygenation for 120 minutes (group D); hypoxia for 45 minutes followed by immediate U-74389G IV administration and reoxygenation for 60 minutes (group E); hypoxia for 45 minutes followed by immediate U-74389G IV administration and reoxygenation for 120 minutes (group F). The dose height selection criteria for EPO and U-74389G were assessed in preliminary studies as 10 mg/kg body mass for both drugs.

Hypoxia was induced by laparotomic clamping of the inferior aorta over the renal arteries with forceps for 45 minutes. Clamp removal restored the inferior aorta patency and reoxygenation. After exclusion of the blood flow, the HR protocol was followed as described above for each experimental group. The drugs were administered at the time