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Association of ABO and Colton Blood Group Gene Polymorphisms With Hematological Traits Variation

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Abstract: Hematological parameters are appraised routinely to determine overall human health and to diagnose and monitor certain diseases. In GWASs, more than 30 loci carrying common deoxyribonucleic acid (DNA) polymorphisms have been identified related to hematological traits. In this study, we investigated the contribution of *ABO* rs2073823 along with *AQP1* rs1049305 and rs10244884 polymorphisms in hematological traits variation in a cohort of Iranian healthy individuals.

Genomic DNA was extracted from peripheral blood of 168 healthy volunteer. Genotyping was performed by ARMS-PCR or PCR-RFLP and confirmed by DNA sequencing. Complete blood analyses were conducted for the participants.

Significant association was observed between *AQP1* rs1049305 and the hematological traits including hemoglobin, hematocrit, and platelet count ($P = 0.012$, 0.008, and 0.011, respectively). The *AQP1* rs10244884 status was also significantly linked to hemoglobin and hematocrit levels in the study cohort ($P = 0.015$ and 0.041, respectively). Furthermore, *ABO* rs2073823 polymorphism was identified as a hemoglobin and hematocrit levels modifier (both with $P = 0.004$).

AQP1 and *ABO* variants appear to predict hemoglobin and hematocrit levels but not other erythrocyte phenotype parameters including red blood cell counts and red blood cell indices.

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Abbreviations: AQP1 = aquaporin-1, ARMS-PCR = amplification refractory mutation system PCR, DNA = deoxyribonucleic acid, Hb = hemoglobin level, Ht = hematocrit, MCV = mean corpuscular volume, PCR = polymerase chain reaction, RBC = red blood cell, RFLP = restriction fragment length polymorphism, SNP = single nucleotide polymorphism.

INTRODUCTION

The presence or absence of certain antigens on the red blood cell (RBC) membrane is generally referred to as term blood group. Some blood group antigens including ABO are carbohydrate structures, found in the glycolipids or glycoproteins located on red cell membrane.[1]

ABO blood group is one of the first identified human molecular polymorphism, composed of 3 common alleles A, B, and O. ABO locus spans more than 18 kilobase (kb) of genomic deoxyribonucleic acid (DNA) on long arm of chromosome 9. It is organized in 7 exons in which exons 6 and 7 are the largest and contain most of the coding sequence.[2] Allelic variations at this locus encode for 2 specific glycosyltransferase: ''A'' that bonds α-*N*-acetylgalactosamine and ''B'' that bonds α-D-galactose to the H acceptor substrate (H antigen). H antigen is encoded by the epistatic H locus on chromosome 19.[3] In O group, the H antigen remains unchanged as a result of premature translation termination and degradation of the A or B truncated glycosyltransferases. In large numbers (but not all) O alleles, the mentioned premature stop codon occurred as a result of a single nucleotide deletion in exon 6 and at amino acid position 261.[1] Two amino acids substitutions, L266M and G268A, in exon 7 determine the A or B specificity of the enzyme in human.[4,5] The ABO locus variants have been correlated with the risk of atherosclerosis,[6,7] coronary heart disease,[8] venous thromboembolism,[9] pancreatic cancer,[10] and plasma levels of coagulation factors.[11–13] This locus has also been related to the biochemical traits such as alkaline phosphatase[14] and hematological parameters such as activated partial thromboplastin time,[15,16] hemoglobin level (Hb),[17,18] hematocrit (Ht),[18] and RBC indices.[18,19] A recent study on Korean population has shown that a minor allele of *ABO* rs2073823 on intron 6 contributes in elevated RBC counts and decreased mean corpuscular volume (MCV).[19]

Colton blood group is determined by the expression of the cell wall water channel, aquaporin-1 (AQP1). AQP1 is involved in the structural modifications of microvasculature and abundantly expressed in erythrocytes and kidney.[20] *AQP1* gene located on 7p14, demonstrates diverse variants with subsequent alteration in the protein expression. It has been shown by luciferase assays that *AQP1* rs1049305 single nucleotide polymorphism (SNP) affects the protein expression level.[21] Deficiency of AQP1 does not produce severe phenotypic consequences. However, it has been observed that *AQP1* gene variations cause mild phenotype of erythrocytes short life span and reduced cell surface.[22] Strong evidence of association was observed between the *AQP1* rs1049305 and water retention in liver cirrhosis.[21] Furthermore, it has been suggested that *AQP1* rs10244884 SNP could predict the risk of vaso-occlusion in sickle cell patients.[23] Considering the effects of *ABO* and *AQP1* gene variants on erythrocyte count, phenotype and indices, we investigated the contribution of rs2073823 variant of *ABO* locus as well as 2 *AQP1* gene variants, that is, 3′UTR rs1049305 and intergenic rs10244884, in the variance of hematological traits in a cohort of Iranian healthy individuals.

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MATERIAL AND METHODS

Subjects

In order to investigate the correlation of *ABO* and *AQP1* gene polymorphisms with hematological traits, we selected 168 healthy individuals based on the review of their past medical records at state health care system as well as the evaluation of their current physical examination findings and routine medical laboratory analysis results. Each participant contributed to the study signed a written consent approved by the ethics committee of the Tarbiat Modares University. Complete blood analyses, including RBC count, platelet number, Hb, Ht, RBC indices, that is, MCV, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were performed for each individual.

DNA Extraction and Genotyping

Peripheral blood sample (2 mL) was obtained from each individual. DNA extraction was performed according to the standard salting out protocol. The concentration and quality of the DNA was measured using NanoDrop (Implen, Germany) ND-1000 spectrophotometer at 260 and 280 nm. DNA samples with the A260%A280 ratios of more than 1.7 were selected for analysis. DNA sample aliquots were stored at −20 °C, and fresh working solutions (10–40 ng/μL) were prepared immediately before each experiment. As indicated in Table 1, specific polymerase chain reaction (PCR) primers for amplification of DNA fragments were designed and verified using SNPs database (dbSNP 129;http://www.ncbi.nlm.nih.gov/projects/SNP/) and Basic Local Alignment Search Tool website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

PCR was performed in a total volume of 20 μL containing approximately 50 ng DNA, 2 μL of 10× PCR buffer, 1.8 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphates, 0.25 pmol of each primer, and 1 U of Taq polymerase. Genomic DNA was amplified by PCR according to the following program: an initial denaturation step for 5 minutes at 94 °C then 30 amplification cycles of – denaturation at 95 °C for 30 seconds, annealing for 30 seconds, and extension at 72 °C for 30 seconds. Final extension was allowed to proceed for 5 minutes at 72 °C. Table 1 shows the respective melting temperatures of each PCR.

The alleles of *ABO* rs2073823 (G/A) were genotyped using tetra-primer amplification refractory mutation system PCR (ARMS-PCR) assay composed of a set of 4 primers (2 allele-specific and 2 common primers). In order to maximize the specificity of allele-specific primers, a mismatch was introduced at the antepenultimate nucleotide of 3′ terminus. PCR product size of common primers that serves as internal control was 613 base pair (bp). Amplification of reverse inner and forward outer primers results in a 394 bp fragment that indicates existence of G allele. In the same way, amplification of forward inner and reverse outer occurs in presence of A allele and results in a 262 bp product.

Genotyping of *AQP1* rs10244884 (T/C) was performed by restriction fragment length polymorphism (RFLP) analysis. Following the amplification of 192 bp fragments, digestions were performed by TaqI endonuclease and verified on a 2% agarose gel. The C allele PCR products were cleaved into 106, 66, and 20 bp; and the G products were cleaved into 172 and 20 bp fragments.

The analysis of *AQP1* rs1049305 (G/C) was performed using a tetra-primer ARMS assay as described previously.[24]

Randomly selected PCR products were subjected to DNA sequencing to verify the RFLP and ARMS results.

Statistical Analysis

The collected data were evaluated by statistical analysis software SPSS V.16. Differences in hematological characteristics and genotype frequencies were determined by Pearson Chi-square (χ^2) analysis. The *P*-values less than 0.05 were considered to be statistically significant. Hardy–Weinberg equilibrium was analyzed using the Genepop web version 4.2 program. Furthermore, separate conditional logistic regression analyses were used to calculate the possible confounding by including sex and age, although no evidence of bias was found.

RESULTS

Hematological Features and Genotyping

Standard CBC analyses were performed on the study participants including 81 males and 87 females. Table 2 describes all blood indices by gender, since gender is known to be related to hematological traits. Males had a higher mean of all indices except for platelet count.

Descriptive data of the 3 SNPs allele frequencies are indicated in Table 3. There was no major deviation from the expected Hardy–Weinberg equilibrium in the subjects allele frequencies. The desired fragments of *ABO* rs2073823, *AQP1* rs1049305, and *AQP1* rs10244884 containing amplicons and

TABLE 1. The Characteristics of the Primers, PCR Product Size, and Annealing Temperature Applied for Each SNP

Target SNP	Primer Name	Oligo Sequence (5′ > 3′)	Product Size	Annealing
*ABO rs*2073823	Forward outer	CTTCCTCCCTCCAGGCTTGA	613 bp	63°
	Reveres outer	AGTGGCGAGTGACTGTGGACA		
	Forward inner	GTGGCTCAGCATGACGGACA	262 bp (A allele)	
	Reverse inner	CCCCTCCTCTCTGTAACTGTGTCC	394 bp (G allele)	
*AQP1 rs*1049305	Forward outer	ACCTGCATGGTCAAGCCTCTTATGGG	657 bp	65°
	Reveres outer	TCTCTGCTTTGTAGCCTGTCTGCTCTGC		
	Forward inner	TGGAATCGTCCCTATATCAGGGCGTC	232 bp (C allele)	
	Reverse inner	TGCCACTTTGCAGAAGGAGGTCAGTC	476 bp (G allele)	
*AQP1 rs*10244884	Forward	ATAGGTGCCACCCATGCTCC	192 bp	61°
	Reverse	GCCTCTGCTCTGCTGACTCG		

bp = base pair, PCR = polymerase chain reaction, SNP = single nucleotide polymorphism.

TABLE 2. Comparison of the Hematological Traits Statistics by Gender

Characteristic and Hematological Traits	Mean	Male (N = 81)	Female (N = 87)	Standard Deviation
Age, year	44.3	47.8	40.8	7.5
RBC, $\times 10^5$/mL	4.81	4.98	4.65	0.4
Hemoglobin, g/dL	13.2	13.9	12.5	1.0
Hematocrite, %	41.0	42.6	39.5	2.1
MCV, fL	84.2	84.8	83.6	3.9
MCH, pg	27.4	27.7	27.1	2.3
MCHC, g/dL	32.5	32.8	31.9	1.3
Platelets, $\times 10^5/\mu$L	2.59	2.43	2.76	0.6

MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, RBC = red blood cell.

their allele-specific fragments were revealed by PCR-RFLP and ARMS-PCR experiments (Figure 1).

ABO rs2073823

As shown in Figure 1, the desired fragments of the tetra-primer ARMS assay for *ABO* rs2073823 SNP were obtained and revealed by gel electrophoresis. The SNP allele frequencies were 0.81 and 0.19 for G and A allele, respectively. Our results showed GG genotype in 65.5% of samples, heterozygote GA genotype in 31.5%, and homozygote AA genotype in 3% of total 168 cases studied. This was in agreement with the expected values due to NCBI website (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?searchType=adhoc_search&type=rs&rs=rs2073823). The statistical data regarding the association of *ABO* rs2073823 to Hb and Ht levels are indicated in Table 4. Interestingly, the minor allele demonstrated a significant correlation with higher levels of Hb and Ht. Higher counts of red cells were observed in AA genotype, although it was not as significant as reported in the study by Hong et al.

AQP1 rs1049305

Tetra-primer multiplex ARMS-PCR for *AQP1* rs1049305 was able to distinguish GG, GC, and CC genotypes. Each PCR reaction produced a control fragment of 657 bp and 2 allele-specific fragments (232 bp of C allele and 476 bp of G allele) based on the genotype of the subject. Both allele-specific fragments were simultaneously present in heterozygote cases. The accuracy of the ARMS assay was confirmed by DNA sequencing of randomly selected representative samples (Figure 1). The allele frequencies of G and C allele were determined 0.55 and 0.45, respectively. Table 5 shows the association studies of rs1049305 genotype and the hematological traits. We observed significant association between this polymorphism and Hb and Ht levels ($P = 0.012$ and 0.008, respectively). Also, platelet count was significantly associated to the rs1049305 genotype ($P = 0.011$). The G allele was related to higher levels of Hb and Ht. However, a lower platelet count was observed to be linked to the G allele.

TABLE 3. The Allele Frequencies Determined for *ABO* rs2073823, *AQP1* rs1049305, and *AQP1* rs10244884 SNPs Among the Study Cohort

Target SNP	Genotype	Frequency, %	Allele Frequency
rs2073823	GG	110 (65.5)	G allele = 0.81
	GA	53 (31.5)	
	AA	5 (3)	A allele = 0.19
	Total	168	
rs1049305	GG	48 (28.5)	G allele = 0.55
	GC	90 (53.5)	
	CC	30 (18)	C allele = 0.45
	Total	168	
rs10244884	TT	50 (30)	T allele = 0.58
	TC	92 (55)	
	CC	26 (15)	C allele = 0.42
	Total	168	

AQP1 = aquaporin-1, SNP = single nucleotide polymorphism.

AQP1 rs10244884

The enzymatic (TaqI) digestion of the amplified fragment of *AQP1* gene containing rs10244884 polymorphism discriminated TT, TC, and CC genotypes. DNA sequencing analysis of representative samples from each genotype confirmed the PCR-RFLP results (Figure 1). The allele frequencies of T and C allele were calculated 0.58 and 0.42, respectively. As indicated in NCBI website the allele frequency of T and C are equivalent in most populations. Table 6 shows the association study of rs10244884 genotype and hematological traits. This SNP status was significantly associated to Hb and Ht levels ($P = 0.015$ and 0.041, respectively). The T allele was related to higher levels of Hb and Ht.

DISCUSSION

Hematological parameters including Hb, Ht, and RBC indices which have potentially high clinical relevance can be influenced by a variety of genetic and environmental factors. Previous studies on twins and different ethnic groups have shown that genetic background contributes to the Hb levels.[25,26] Further evidence derived from linkage analysis showed joint linkage of Ht and Hb to chromosome 9q34 with candidate *EBP41L2* and *HEBP2* genes.[27] Although there is a lack of replication for these linkages as several other loci such as *HBS1L/MYB*, *TMPRSS6*, *TFR2*, *TFRC*, and *SH2B3* were also linked to Hb and Ht in the CHARGE consortium study.[28,29] The rs5756506 SNP located on *TMPRSS6* was considered as the only RBC locus strongly associated with Hb levels, reported by a genome-wide meta-analysis.[28] The HFE rs1800562 (Cys282 Tyr) which is rare in African populations was also reported to be

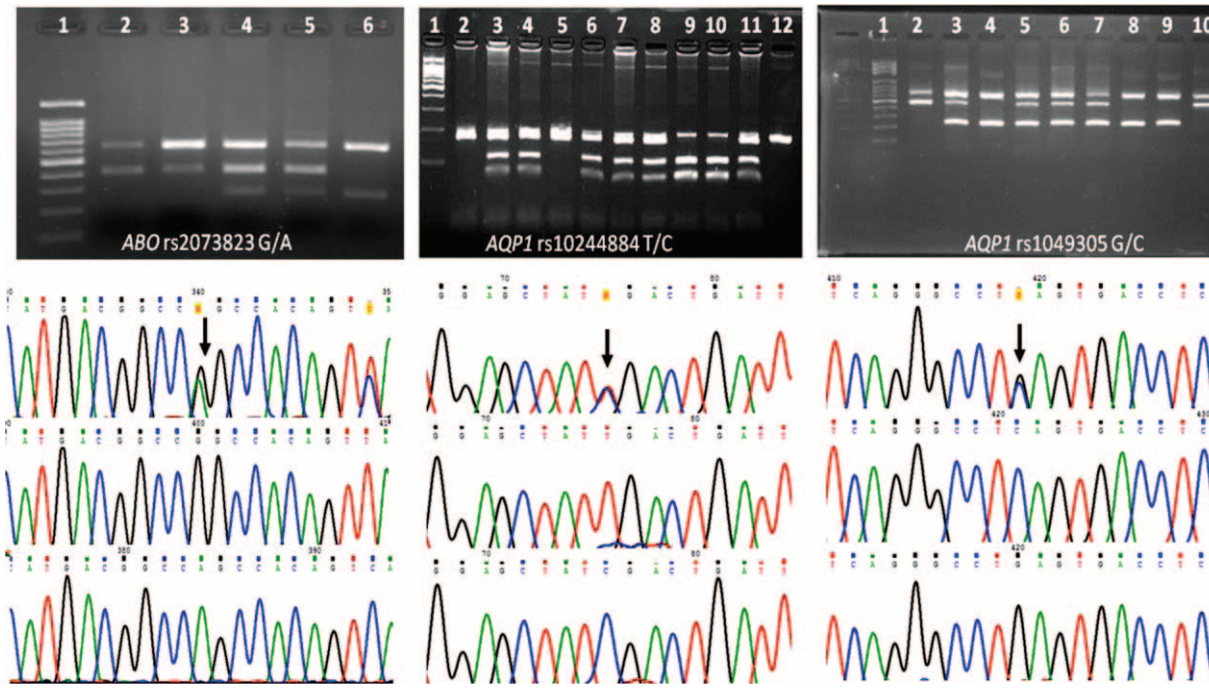

FIGURE 1. Genotyping results. Left – *ABO* rs2073823 tetra-primer ARMS and sequencing. Lane 1, 100 bp ladder, lanes 2 and 3 present GG genotype, lanes 3 and 4 indicate GA genotype, lane 6 shows AA genotype. Middle – *AQP1* rs10244884 PCR-RFLP and sequencing. Lane 1, 100 bp ladder, lanes 2, 5, and 12 indicate TT genotype, lanes 3, 4, 6, 7, 8, and 11 show TC genotype, and lanes 9 and 10 CC genotype. Right – *AQP1* rs1049305 tetra-primer ARMS and DNA sequencing. Lane 1, 100 bp ladder, lanes 2 and 10 present GG genotype, lanes 3, 5, 6, and 7 GT genotype, lanes 4, 8, and 9 show TT genotype. AQP1 = aquaporin-1, ARMS = amplification refractory mutation system, DNA = deoxyribonucleic acid, PCR = polymerase chain reaction, RFLP = restriction fragment length polymorphism.

involved in Hb and Ht modifications.[29] The diversity of candidate genes, bring the idea of population-specific manner of gene polymorphisms, effects on hematological traits.

In our study, *ABO* rs2073823 SNP showed a significant association with Hb and Ht in Iranian population. In a cohort of Korean people 6 tagging SNPs (rs2073823, rs8176720, rs495828, rs2073823, rs8176717, and rs687289) were analyzed related to hematological traits including white blood cell count, RBC, platelet, MCV, and mean corpuscular hemoglobin concentration. They reported a strong association between *ABO* rs2073823 and MCV as well as RBC. Since ABO rs2073823 is in perfect LD ($r2 = 0.995$) with rs8176746, a deterministic variant of the B-type blood group, they concluded that type B blood group might increase RBC counts in comparison to other blood types.[19] Further elucidating of ABO genotype may provide a more accurate representation of the influence of ABO on blood indices by identifying heterozygous individuals.

To our knowledge, there has been no report on the direct association between *AQP1* gene polymorphism and hematological traits. However, the variants of this gene have been linked to phenotype of reduced red cell surface area and short lifespan of erythrocytes in human.[22] The rs10244884 is located at intergenic area, downstream to *AQP1* gene. Intergenic polymorphisms have been widely studied related to pathological conditions. It has been suggested that intergenic SNPs may contribute in the regulation of adjacent genes.

The *AQP1* rs1049305 was studied in a group of marathon runners. They found a significant association between this SNP

TABLE 4. The Results of Association Analysis Between Hematological Traits and *ABO* rs2073823 Polymorphism

	rs2073823 GG Mean (SD)	rs2073823 GA Mean (SD)	rs2073823 AA Mean (SD)	*P*-Value
RBC, $\times 10^5$/mL	4.82 (0.37)	4.84 (0.40)	5.05 (0.15)	0.591
Hemoglobin, g/dL	12.97 (0.98)	13.32 (1.13)	14.96 (0.64)	**0.004**[*]
Hematocrite, %	40.32 (2.00)	41.28 (2.27)	44.06 (0.77)	**0.004**[*]
MCV, fL	83.90 (5.45)	85.02 (5.16)	87.70 (2.16)	0.379
MCH, pg	26.84 (2.95)	27.62 (2.57)	29.83 (1.80)	0.140
MCHC, g/dL	32.17 (1.51)	32.44 (1.60)	33.96 (1.28)	0.136
Platelets, $\times 10^5/\mu$L	2.70 (5.98×10^4)	2.39 (4.94×10^4)	2.91 (7.60×10^4)	0.065

MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, RBC = red blood cell, SD = standard deviation.

[*] Statistically significant difference between the values in the subjects with different genotypes (*P* value <0.05).

TABLE 5. The Results of Association Analysis Between Hematological Traits and *AQP1* rs1049305 Polymorphism

Hematological Traits	rs1049305 GG Mean (SD)	rs1049305 GC Mean (SD)	rs1049305 CC Mean (SD)	*P*-Value
RBC, $\times 105$/mL	4.84 (0.23)	4.84 (0.41)	4.78 (0.46)	0.886
Hemoglobin, g/dL	13.54 (0.98)	13.13 (1.05)	12.50 (1.05)	**0.012***
Hematocrite, %	41.36 (1.89)	40.87 (2.21)	39.21 (2.09)	**0.008***
MCV, fL	85.40 (2.40)	84.59 (5.49)	82.45 (7.19)	0.222
MCH, pg	27.52 (2.72)	27.30 (2.75)	26.40 (3.10)	0.453
MCHC, g/dL	32.69 (1.17)	32.23 (1.64)	31.96 (1.64)	0.303
Platelets, $\times 105/\mu L$	2.33 (4.17×10^4)	2.65 (5.77×10^4)	2.87 (6.78×10^4)	**0.011***

MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, RBC = red blood cell, SD = standard deviation.
* Statistically significant difference between the values in the subjects with different genotypes (*P* value <0.05).

TABLE 6. The Results of Association Analysis Between Hematological Traits and *AQP1* rs10244884 Polymorphism

Hematological Traits	rs10244884 TT Mean (SD)	rs10244884 TC Mean (SD)	rs10244884 CC Mean (SD)	*P*-Value
RBC, $\times 10^5$/mL	4.87 (0.28)	4.83 (0.39)	4.75 (0.47)	0.635
Hemoglobin, g/dL	13.59 (1.05)	13.05 (1.08)	12.58 (0.81)	**0.015***
Hematocrite, %	41.36 (2.28)	40.72 (2.11)	39.46 (1.98)	**0.041***
MCV, fL	84.96 (2.72)	84.40 (6.03)	83.59 (6.02)	0.747
MCH, pg	27.92 (1.55)	26.95 (3.31)	26.74 (2.63)	0.313
MCHC, g/dL	32.84 (1.27)	32.12 (1.66)	31.97 (1.34)	0.116
Platelets, $\times 10^5/\mu L$	2.50 (6.08×10^4)	2.60 (5.55×10^4)	2.76 (6.29×10^4)	0.443

MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, RBC = red blood cell, SD = standard deviation.
* Statistically significant difference between the values in the subjects with different genotypes (*P* value < 0.05).

and the runner's action. They concluded that, as a 3′ UTR polymorphism, *AQP1* rs1049305 in an interaction with micro-ribonucleic acids could influence the messenger ribonucleic acid expression and AQP1 protein levels.[24] This protein is considered as a factor of cell viability improvement, and an over expression of *AQP1* has been described in diverse types of human cancers.[30] Recently, using adeno-associated vector carrying *AQP1*, a gene therapy has been done to treat radiation-induced salivary gland hypofunction in Balb/c mice. Hematological traits variation was reported for Ht, although it was limited to the highest dose.[31]

In conclusion, our study provides insight into the putative role for *ABO* rs2073823, *AQP1* rs1049305, and *AQP1* rs10244884 gene polymorphisms in variance of Hb and Ht levels. Although, partly limited by sample size, this study showed that these SNPs could develop the inter population variation. A more complete elucidation of the blood indices disparity will allow more accurate analyses and improve estimates of their clinical significance.

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