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.

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.

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. Allergic 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. 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. Two amino acid substitutions, L266M and G268A, in exon 7 determine the A or B specificity of the enzyme in human. The ABO locus variants have been correlated with the risk of atherosclerosis, coronary heart disease, venous thromboembolism, pancreatic cancer, and plasma levels of coagulation factors. This locus has also been related to the biochemical traits such as alkaline phosphatase and hematological parameters such as activated partial thromboplastin time, hemoglobin level (Hb), hematocrit (Ht), and mean corpuscular volume. SNP = single nucleotide polymorphism.

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.
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 MgCl2, 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 rs1049305 (G/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 (χ²) 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

| Target SNP   | Primer Name | Oligo Sequence (5’ > 3’) | Product Size | Annealing |
|--------------|-------------|--------------------------|--------------|-----------|
| ABO rs2073823 | Forward outer | CTCCCTCCCTCCAGGCTTTGA | 613 bp       | 63°C      |
|              | Reverses outer | AGTGGCAGTGACTGTGGACAC |             |           |
|              | Forward inner | GTGGCTCAGCATGACGGACA | 262 bp (A allele) |           |
|              | Reverse inner | CCCCTCCTCTGTAACCTGTGCC | 394 bp (G allele) |           |
| AQP1 rs1049305 | Forward outer | ACCTCGATGTCGAACGCTTTATGG | 657 bp | 65°C |
|              | Reverses outer | TCTCTGCTTTTGTAGCGCTGCC |           |           |
|              | Forward inner | TGGAGCTTGCTCTATATCGCGCT | 232 bp (C allele) |           |
|              | Reverse inner | GCCAGACTTGGCAGAGGAGTGCCT | 476 bp (G allele) |           |
| AQP1 rs10244884 | Forward | ATAGTGCCACCCATGTTC | 192 bp | 61°C |
|              | Reverse | GCCCTTGTCTGTGCTACGC |           |           |

bp = base pair, PCR = polymerase chain reaction, SNP = single nucleotide polymorphism.
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&type¼rs&rs). 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.

**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. Further evidence derived from linkage analysis showed joint linkage of Ht and Hb to chromosome 9q34 with candidate EBP4IL2 and HEBP2 genes. Although there is a lack of replication for these linkages as several other loci such as HBS1L/MBY, TMPRSS6, TF2R, TFRC, and S2H23 were also linked to Hb and Ht in the CHARGE consortium study. 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. The HFE rs1800562 (Cys282 Tyr) which is rare in African populations was also reported to be

### 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, ×10^7/mL                          | 4.81 | 4.98         | 4.65           | 0.4               |
| Hematocrite, %                         | 13.2 | 13.9         | 12.5           | 1.0               |
| Hemoglobin, g/dL                       | 41.0 | 42.6         | 39.5           | 2.1               |
| MCV, fL                                | 84.2 | 84.8         | 83.6           | 3.9               |
| MCH, pg                                | 27.5 | 27.7         | 27.1           | 2.3               |
| MCHC, g/dL                             | 32.5 | 32.8         | 31.9           | 1.3               |
| Platelets, ×10^9/μL                    | 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.

### 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)    | A allele = 0.19  |
|            | AA       | 5 (3)        |                  |
|            | 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.
involved in Hb and Ht modifications.\textsuperscript{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 ($r^2 = 0.995$) 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.\textsuperscript{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.\textsuperscript{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 and hematological traits.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{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.}
\end{figure}

\begin{table}
\centering
\caption{The Results of Association Analysis Between Hematological Traits and ABO rs2073823 Polymorphism}
\begin{tabular}{lllll}
\hline
 & rs2073823 GG Mean (SD) & rs2073823 GA Mean (SD) & rs2073823 AA Mean (SD) & \textit{P}-Value \\
\hline
RBC, $\times 10^{12}$/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, $\times 10^{12}$/μL & 2.70 (5.98 $\times 10^{4}$) & 2.39 (4.94 $\times 10^{4}$) & 2.91 (7.60 $\times 10^{4}$) & 0.065 \\
\hline
\end{tabular}
\end{table}

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, ×10^5/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*  |
| Hematocrit, %        | 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, ×10^4/μL  | 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, ×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*  |
| Hematocrit, %        | 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, ×10^4/μL  | 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 microribonucleic 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 inducing AQP1 expression and AQP1 protein levels.\(^24\) This protein is "induced salivary gland hypofunction in Balb/c mice. Hematological traits variation was reported for Ht, although it was induced salivary gland hypofunction in Balb/c mice. Hematological traits variation was reported for Ht, although it was induced salivary gland hypofunction in Balb/c mice. Hematological traits variation was reported for Ht, although it was induced salivary gland hypofunction in Balb/c mice."

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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