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PHENOTYPIC AND GENOTYPIC ASSOCIATION OF THE DUFFY BLOOD GROUP ANTIGENS (FYA, FYB) IN HEALTHY BLOOD DONORS OF TABUK –SAUDI ARABIA

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ABSTRACT
Duffy encoded by the ACKR1 gene is a blood group system and glycoprotein antigens found on RBCs, endothelial cells of blood vessels, and capillaries of glomeruli, peri-bronchioles, and Purkinje cells in the central nervous system. Duffy blood system was reported to be associated with malaria infection, neutropenia, chronic infections, hemolytic disease of the fetus and newborn (HDFN) and acute or delayed hemolytic blood transfusion reactions. In this study, we analyzed the Duffy blood group system using the amplification mutation system PCR (ARMS-PCR) in blood donors (200, males and females) from Tabuk and Duba region. Results showed that the c. 125 G>A SNP genotype distribution was AA (Fy a- b+) genotype (51 vs. 49%), GA (Fya+ Fyb+) genotype (40 vs. 40%) followed by GG (Fya+ Fyb-) (9 vs. 11%). Analysis of the c.265C>T SNP was CC genotype (97 vs. 98%), CT genotype (3 vs. 2%) followed by TT (0 vs. 0%). The c.1-67T>C SNP genotype distribution was CC genotype (44 vs. 43%), CT genotype (41 vs. 37%) followed by TT (20 vs. 15%). The CC genotype of the c.1-67T>C abolishes Fy antigen expression (FY*Null) is relatively high. This result shed light on the Duffy antigen distribution in North region of KSA but requires further validation in future studies with larger sample size.

KEYWORDS: Blood group, ACKR1 gene, amplification refractory mutation system PCR, Duffy antigen receptor for chemokines

INTRODCTION
Duffy (also called the Duffy antigen) receptor for chemokines (DARC), or Fy glycoprotein, is a glycoprotein receptor that binds chemokines [1-3]. The Duffy glycoprotein with a molecular weight 40 KD is present on the surface of the erythrocytes, endothelial cells of blood vessels, and capillaries of glomeruli, peri-bronchioles, and Purkinje cells in the central nervous system [4]

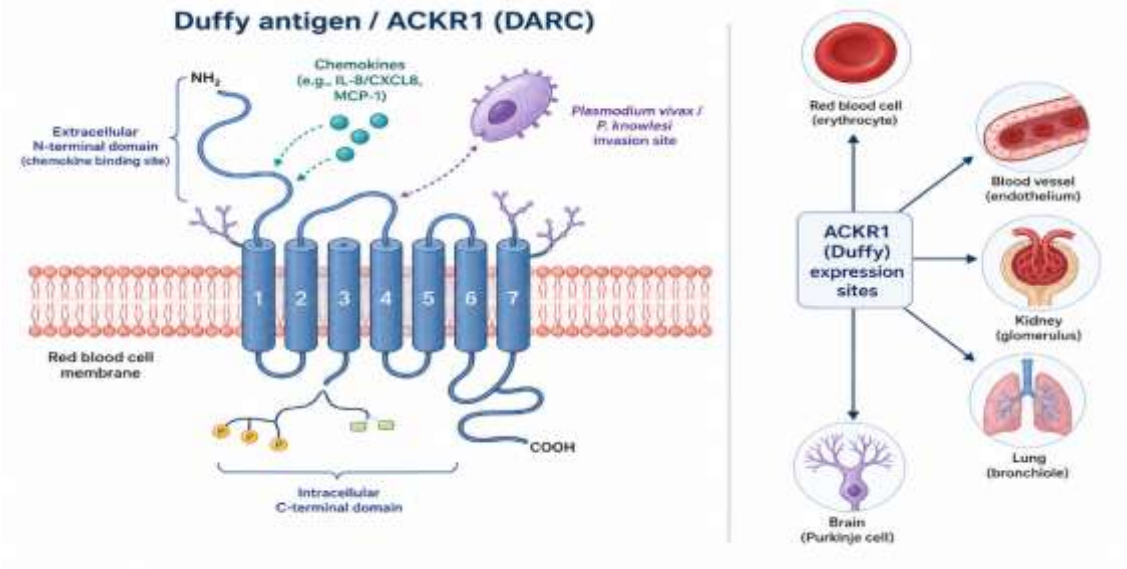


Figure 1: Schematic representation of the Duffy antigen receptor (ACKR1/DARC), showing the extracellular N-terminal domain (chemokine and malaria parasite binding site), the intracellular C-terminal domain, and the tissue distribution of ACKR1 expression.

Duffy glycoprotein is nonspecific receptor for certain cytokines, and is homologous to the superfamily of the G-protein chemokine receptor, which is proposed to work as a scavenger in inflammatory-and proinflammatory-related disorders [5]. Duffy is a single copy gene found on chromosome 1 (1.q22-1.q23) [5]. Duffy regulates the homeostatic levels of the pro-inflammatory chemokines [6]. It maintains the gradient of chemokine between the blood and the tissues such that the neutrophils and monocytes move from blood vessels into tissues [6]. The Duffy (Fy) antigen constitutes seven-transmembrane glycoprotein on cell membranes [7]. Its role is not fully elucidated, but it is atypical chemokine receptor (ACKR1), and also acts as a receptor for malaria parasites (Plasmodium vivax and Plasmodium knowlesi), HIV-1 and tetraspanin CD82 [1, 6, 8, 9]. The tetraspanins are proteins family assembles protein complexes at the cellular membrane, and tetraspanin CD82 is important for muscle function and myofiber[10]. The Duffy glycoprotein is composed of an extracellular amino-terminal domain and an intracellular C-terminal domain. It binds the chemokines (C-C-L and C-X-C-L) via the extracellular amino terminal domain [5]. Duffy also called the cluster of differentiation 234 (CD234) or Fy-glycoprotein (Fy)[1]. The main Fy antigens are Fya and Fyb are the two main co-dominant alleles resulted from the 125 G>A single nucleotide polymorphism (SNP) leads to one amino acid exchange, Glycine 42 Aspartate, 125G>A[1] The expression of these polymorphic antigens results in various Fy phenotypes: Fy (a+b+), Fy (a+b-) and Fy (a-b+)[1]. The Duffy blood group encoded by the ACKR1 gene is highly variable with the distribution of alleles varying among different populations and ethnicities [11]. For example, ACKR1 gene variations modulate the risk to Plasmodium vivax and affect the humoral immunity to plasmodium antigens [12]. Moreover, it has been reported that the Duffy plus interleukin-8 gene variations were associated with chronic periodontitis in Brazilian population [9]. The Duffy antibodies (Fy a and –Fyb) emerge because of exposure to Duffy antigens through pregnancy and result in hemolytic disease of the fetus and newborn (HDFN) [13, 14]. Moreover, anti-Duffy antibodies (Fy3) resulted in acute or delayed hemolytic blood transfusion reactions[14]. Furthermore, Duffy blood group alleles and genotypes can be used as anthropological markers[15]. The aim of the current study was to genotype the antigens of Duffy blood group, and to examine the frequency of Duffy alleles in subjects from Tabuk and Duba cities, northwestern kingdom Saudi Arabia.

METHODOLOGY:

Study population: This study was conducted at the Prince Fahd Bin Sultan Research Chair for Biomedical Research, University of Tabuk, where participants were enrolled from the hematology outpatient department of King Fahad Specialist Hospital, Tabuk. Written informed consent was obtained from every participant prior to blood sample collection.

Inclusion and exclusion criteria: : Blood samples were obtained from apparently healthy donors, aged 18-60 years, attending the blood bank of the hematology OPD at King Fahad Specialist Hospital, Tabuk. Individuals presenting with any significant underlying illness were not enrolled in the study.

Genomic DNA extraction: : Peripheral venous blood (3 ml) was drawn from each donor into EDTA-coated tubes. Genomic DNA was isolated from these samples with the DNeasy Blood Kit (Qiagen, Germany), following the manufacturer's protocol, and the resulting DNA was resuspended in 200ul of TE buffer. DNA integrity was assessed by 1% agarose gel electrophoresis, while sample purity was confirmed using a NanoDrop™ spectrophotometer (Thermo Scientific, USA).

Genotyping of ACKR1 rs12075 A>G, ACKR1 rs2814778 T>C, ACKR1 rs34599082 C>T: Genotyping for the three ACKR1 variants (rs12075 A>G, rs2814778 T>C, rs34599082 C>T) was performed using an optimized ARMS-PCR protocol [16-19], with primers designed in Primer3 software (Table 1). Each ARMS-PCR reaction was set up in a total volume of 25 uL, comprising 60 ng of template DNA, 7 uL of GoTaq® Green Master Mix (cat no. M7122, Promega, USA), and 0.12 uL each of the forward outer (Fo), reverse outer (Ro), forward inner (FI), and reverse inner (RI) primers.

Table1: ARMS PCR primers ACKR1 rs12075 A>G , ACKR1 rs2814778 T>C , ACKR1 rs34599082 C>T genotyping

ARMS PCR primer ACKR1 rs12075 A>G (of c.125G>A (rs12075)			
ACKR1Fo	5'-GAGACCTTGTTCTCCCACCCGACCTT-3'	60°C	370 bp
ACKR1Ro	5'-AGCTGCCAGCGGAAGAGAGGTCTGAAAA-3'		
ACKR1-GFI	5'-GAATGATTCCTTCCCAGATGGAGACTAGGG-3'		180 bp
ACKR1-ARI	5'-GGGGCAGCTGCTTCCAGGTTGGAAT-3'		244 bp
ARMS PCR primer ACKR1 rs2814778 T>C rs2814778 (−67 T > C)			
ACKR1Fo	5'-ACAGGAAGACCCAAGGCCAGTGA-3'	61°C	391bp
ACKR1Ro	5'-AGGAGGCTAGCATAGGAAGGAGA-3'		
ACKR1-AFI	5'-CCCTCATTAGTCCTTGGCTCTTA-3'		284bp
ACKR1-CRI	5'-TCAGCGCCTGTGCTTCCAAGAG-3'		151bp
ARMS PCR primer ACKR1 rs34599082 C>T c.265C>T, rs34599082			
ACKR1Fo	5'-CTCAACTGAGAACTCAAGTCAGCTGGA-3'	62°C	388 bp

ACKR1Ro	5'-AAAGGCTGAGCCATACCAGACAC-3'		
ACKR1-CFI	5'-CTTCATGCTTTTCAGACCTCTCTTCC-3'		184bp
ACKR1-TRI	5'-AGGGCAGAGCTGCCAGCA-3'		247 bp

Thermocycling conditions: An optimal annealing temperature for each variant was established using a gradient PCR approach tested across a 55°C to 64°C range, which typically achieved optimization within a single trial. This yielded annealing temperatures of 60°C for ACKR1 rs12075 A>G, 61°C for ACKR1 rs2814778 T>C, and 62°C for ACKR1 rs34599082 C>T (with 60°C used as the general genotyping annealing setting on the gradient PCR thermocycler). Increasing the cycle number from 35 to 45 markedly improved the yield of all three PCR products; agarose gel electrophoresis showed that this adjustment produced stronger amplification of the mutant allele relative to the control. The final cycling protocol consisted of an initial hot start at 95°C for 7 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at the variant-specific temperature for 35 seconds (Table No. 1), and extension at 72°C for 40 seconds, with a final elongation step at 72°C for 8 minutes and storage at 4°C.

Statistical analysis: Duffy allele frequencies in the Tabuk cohort were compared with those in the Duba cohort using an odds-ratio approach, supplemented by two-tailed Fisher-Exact or Chi-Squared tests as appropriate. Associations for the ACKR1 rs12075 A>G, rs2814778 T>C, and rs34599082 C>T variants were expressed as odds ratios with 95% confidence intervals. All statistical analyses were carried out in GraphPad version 8.0 and SPSS version 25, with statistical significance defined as a p value of less than 0.05.

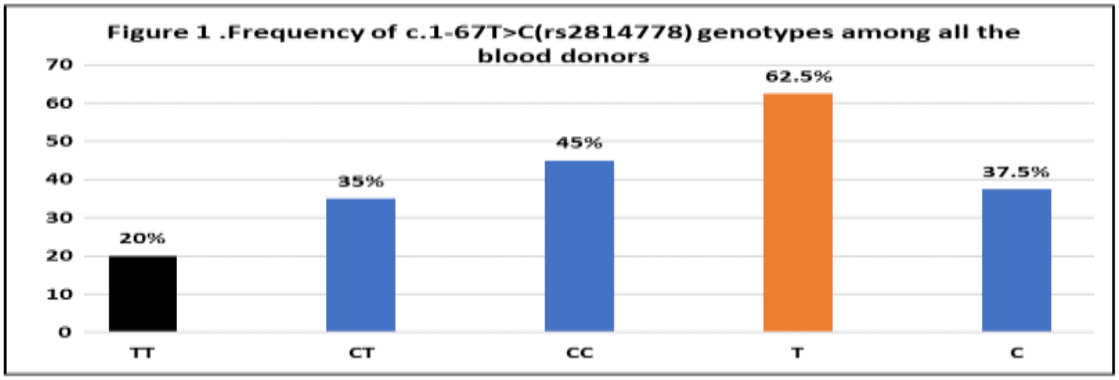
RESULTS

Allele and genotype frequencies of rs2814778 (−67T>C): Table 2 and Figure 1 summarize the allele and genotype distribution of rs2814778 (−67T>C) across the 200 samples analyzed. Among the two alleles, T was more frequent than C (62.5% vs. 37.5%; Table 2). A regional comparison further revealed that the T allele occurred more often among Tabuk donors than Duba donors (64.5% vs. 61.5%).

Table2: Distribution of Allele and genotype frequencies of rs2814778 (−67 T > C) in samples

Polymorphism	Genotype	All blood donors	Tabuk Blood donors	Duba Blood donors	OR (95% CI)	P value
		N=200	**100**	**100**		
rs2814778 (−67 T > C)	**T**	250(62.5)	129(64.5%)	123(61.5%)	**Ref (1)**	
	C	150(37.5%)	71(35.5%)	77(38.5%)	1.13 (0.7577 to 1.7074)	0.53
	TT	40(20%)	15(15%)	20(20%)	Ref (1)	
	CT	70(35%)	41(41%)	37(37%)	0.93(0.4996 to 1.7381)	0.82
	CC	90(45%)	44(44%)	43(43%)	1.08(0.5874 to 1.9964)	0.79

At the genotype level, the CC genotype of c.1-67T>C (rs2814778) — associated with abolished Fy antigen expression (FY*Null) — accounted for 45% of donors (Table1), while the heterozygous TC and homozygous wild-type TT genotypes made up 35% and 20% of the remaining samples, respectively (Table 2).



No significant difference in c.1-67T>C genotype prevalence was observed between the Tabuk and Duba cohorts (P-value > 0.05; Table 2). Both the T allele and the CC genotype were dominant overall (OR = 0.83 [0.3815 to 1.8202]; Table 2), and although the T allele was marginally more frequent in Tabuk than Duba samples, this difference did not reach statistical significance (p<0.64). The TT genotype remained dominant in both groups (64.5 and 61.5%, respectively; Table 2).

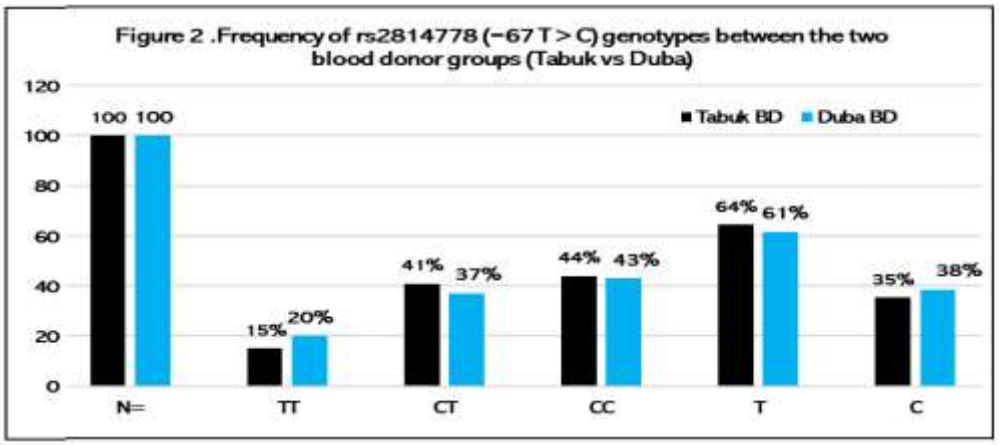


Figure 2: The c.1-67T>C (rs2814778) SNP genotype distribution in all blood donors from Tabuk and Duba. Allele and genotype frequencies of rs12075 (125G>A): For c.125G>A (rs12075), allele and genotype data across all 200 samples are presented in Table 3 and Figure 3. The G allele predominated over the A allele overall (72.5 and 27.7%, respectively; Table3), and this G-allele frequency was marginally higher among Tabuk donors than Duba donors (71% vs 69%).

Table3: Distribution of Allele and genotype frequencies of rs12075 (125G>A) in samples							
Polymorphism	**Genotype**		**All blood donors**	**Tabuk Blood donors**	**Duba Blood donors**	**OR (95% CI)**	**P value**
rs12075 (125G>A)	**N**		N=200	N=100	N=100		
	G		290(72.5%)	142(71%)	138(69%)	**Ref (1)**	
	A		110(27.7%)	58(29%)	62(31%)	1.10(0.7170 to 1.687)	0.66
	GG	FY*A	20(10%)	9(9%)	11(11%)	**Ref (1)**	
	GA	FY*AB	70(35%)	40(40%)	40(44%)	0.81(0.305 to 2.188)	0.68
	AA	FY*B	110(55%)	51(51%)	49(49%)	0.78(0.2997 to 2.0618)	0.62

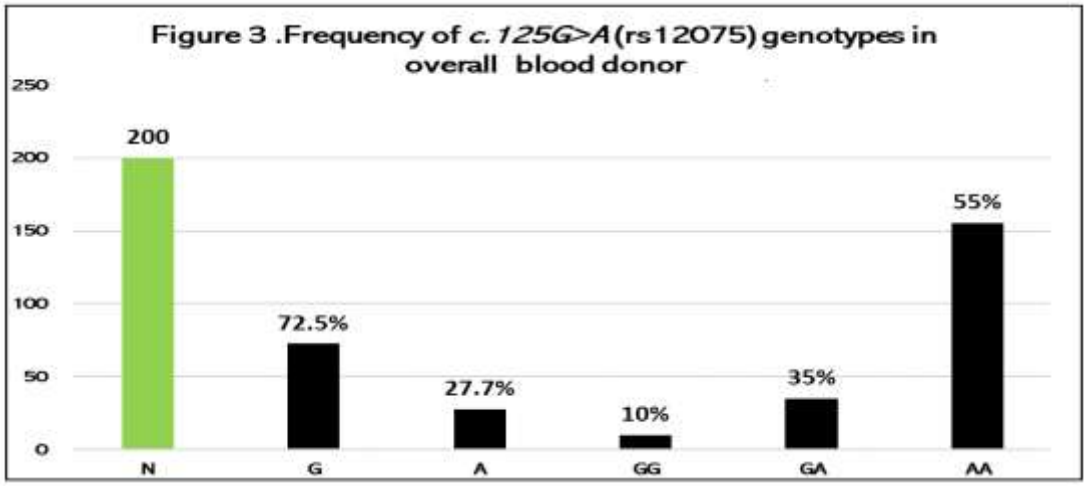


Figure 3: The rs12075 (125G>A) SNP genotype distribution in all blood donors. At the genotype level, the AA genotype (FY*B) was the most common, present in 55% of samples, followed by the heterozygous GA genotype (FY*AB) at around 35% and the homozygous GG genotype (FY*A) at 10% (Table3). Genotype frequencies for rs12075 (125G>A) did not differ between the Tabuk and Duba groups (P-value = 0.66; Table 3). The G allele was found to be dominant within the AA genotype (OR = 1.10 [0.7170 to 1.687], p<0.66), and the AA genotype remained the most frequent in both regions (51% and 49%, respectively; Table 2, figure 4).

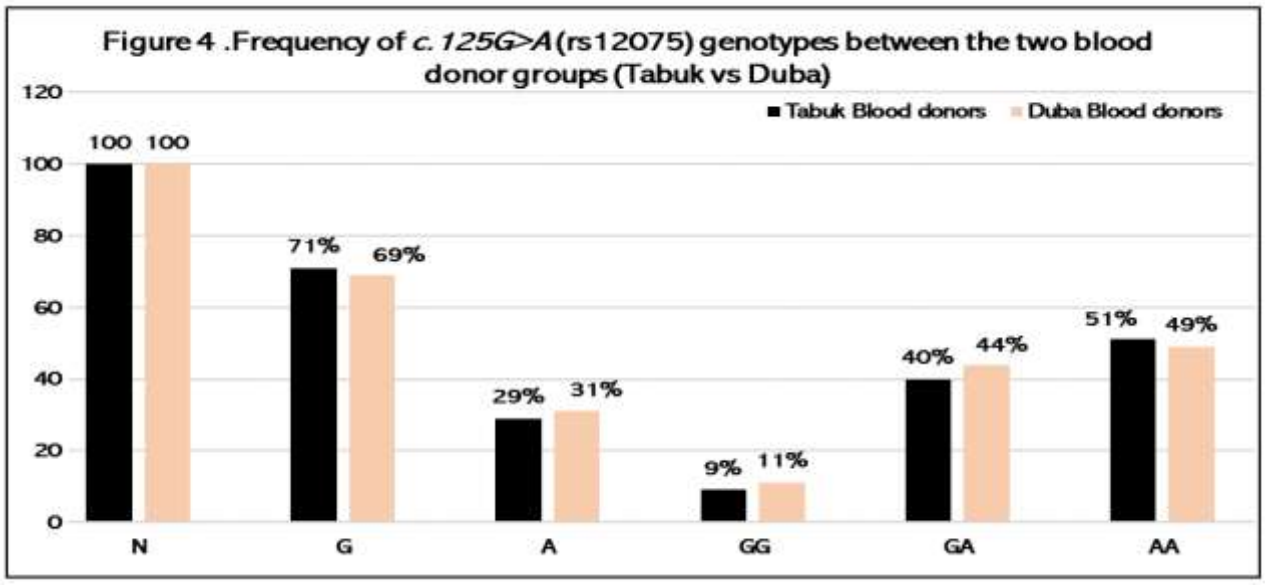


Figure 4: The rs12075 (125G>A) SNP genotype distribution in all blood donors from Tabuk and Duba .

Genotype distribution of c.265C>T, rs34599082 C>T, p.Arg89Cys in samples

3. Genotype distribution of c.265C>T, rs34599082 C>T, p.Arg89Cys: Allele and genotype frequencies for this variant across the 200 samples are presented in Table 4. The C allele was overwhelmingly more frequent than the T allele (99% and 1%, respectively; Table4), and this C-allele frequency was slightly lower in Tabuk than in Duba donors (98.5 vs. 99%). Correspondingly, the CC genotype accounted for 98% of samples, with the remaining donors carrying the heterozygous TC genotype (2%) and no donors carrying the TT genotype (0%; Table4). Genotype frequencies for c.265C>T did not differ significantly between the Tabuk and Duba groups (P-value= 0.66; Table 4). Both the C allele and CC genotype were dominant overall (OR = 0.32 (0.0334 to 3.19)), p<0.33, and although the C allele was somewhat lower in Tabuk relative to Duba samples (p<0.66), the TT genotype remained virtually absent in both groups (Table 4).

Table4: Distribution of Allele and genotype frequencies of c.265C>T, rs34599082 C>T, p.Arg89Cys in samples

Polymorphism	Genotype	All blood donors	Tabuk Blood donors	Duba Blood donors	OR (95% CI)	P value
		N=200	100	100		
c.265C>T, rs34599082 C>T	C	396(99%)	197(98.5%)	198(99%)	**Ref(1)**	
	T	4(1%)	3(1.5%)	2(1%)	0.66(0.1096 to 4.013)	0.43
	CC	196(98%)	97(97%)	99(98%)	**Ref(1)**	
	CT	4(2%)	3(3%)	1(2%)	0.32(0.0334 to 3.19)	0.33
	TT	0	0	0		

Duffy allele genotypes frequencies in northwestern Saudi Arabian blood donors in comparison to other population frequencies, obtained from 1000 genomes database. 4. Comparison with 1000 Genomes population data: Table 5 presents the allele and genotype frequencies of northwestern Saudi Arabian donors alongside global population frequencies drawn from the 1000 Genomes Database. Allele frequencies for c.1-67T>C, c.125G>A, and c.265C>T in this cohort differed substantially from the pooled frequencies across all reference populations (P <0.001; Table 5), whereas genotype frequencies for the same three SNPs were comparable to the overall genotype rates observed across populations (P-value <0.001). Contingency-table comparisons between the study cohort and the 1000 Genomes reference populations were performed using two-tailed 2X2 or 3X2 chi-squared tests, with the 2X2 Fisher exact test applied instead whenever an expected genotype or allele frequency in the contingency table was less than five (*).

Table5: Duffy allele frequencies and genotypes in Western Saudi Arabian blood donors in comparison to other population frequencies, obtained from 1000 genomes database

Polymorphism rs2814778 (−67 T > C)	**Allele**	**Genotype**	**Northwestern Region (Saudi Arabia)**	**All populations**	**X2**	P value
	T		250(62.5%)	3674 (73.4%)	21.41	0.0001

	C		150(37.5%)	1334 (26.6%)		
		TT	40(20%)	1793 (71.6%)	415.15	0.001
		CT	70(35%)	88 (3.5%)		
		CC	90(45%)	623 (24.9%)		
Polymorphism (125G>A) rs12075	**Allele**	**Genotype**	**Northwestern Region (Saudi Arabia)**	**All populations**	**X2**	**P value**
	G		290(72.5%)	2707 (54.1%)	50.27	0.0001
	A		110(27.7%)	2301 (45.9%)		
		GG	20(10%)	794 (31.7%)	42.15	0.0001
		GA	70(35%)	713 (28.5%)		
		AA	110(55%)	997) 28.5%(		
Polymorphism c.265C>T, rs34599082 C>T	**Allele**	**Genotype**	**Northwestern Region (Saudi Arabia)**	**All populations**	**X2**	**P value**
	C		396(99%)	4985) 99.5%(	1.23	0.26
	T		4(1%)	23 (0.5%)		
		CC	196(98%)	2481 (99.1%)	2.19	0.334
		CT	4(2%)	23 (0.9%)		
		TT	0	0 (0%)		

DISCUSSION

Duffy antigen receptor for chemokines (DARC) or Duffy antigen is a glycoprotein found in membrane of erythrocyte and other cells. The Duffy antigen is encoded by DARC gene (or ACKR1 Gene) which is located on chromosome 1[20]. The FYA (G125) and FYB (125A) alleles are produced by the common Duffy gene variant G125A (rs12075) [2]. The wildtype FYA/FYA (corresponding to the phenotype Fy (a+, b−), FYA/FYB (Fy (a+, b+)), and FYB/FYB (Fy (a−, b+)) are the genotypes for the Duffy antigens [2]. A single base substitution in the DARC gene promoter region (T-33C) blocks Duffy antigen expression and resulted in negative Duffy blood group (Fy (a−, b−)) [2]. In addition, the single nucleotide variations (SNVs), c.265C>T (rs34599082) and c.298G>A (rs13962), cause an exchange of Arg to Cys at position 89 amino acid residue (p.Arg89Cys), and an Alanine to Threonine substitution at the amino acid residue100 (p.Ala100Thr) of Duffy glycoprotein, respectively[1]. These two SNVs leads to faint expression of Fyb and called FY*02W.01 allele or Fyx antigen)[1].

Result showed that the genotype distribution of the c.1-67T>C (rs2814778) SNP were 45% CC, 20% TT and CT 35%. (Table1, Figure1). Our result showed that there was no significant differences in genotype distribution between Tabuk and Duba blood donor (Figure2). The CC genotype abolishes Fy antigen expression (FY*Null or Duffy-null)[21]. It has been reported that individuals with the Duffy-null genotype had a lower neutrophil count than individuals with TC or TT genotype of the c.1-67T>C (rs2814778) SNP. That is the c.1-67T>C (rs2814778) was reported to be associated with benign constitutional neutropenia [25].

Nevertheless, Duffy-null genotype is not linked to elevated risk for bacterial of viral infection [21]. The CC genotype is not linked to elevated risk of infection or other diseases. However, there is a medical consequence of this decreased WBCs count [22, 23]. For instance, carriers CC genotype of the c.1-67T>C (rs2814778) SNP were more likely to perform a bone marrow biopsy for detected decreased WBCs count than the CT/TT genotypes carriers, and more than 95% of their biopsies had normal results[23, 24]. In addition, the rs2814778-C SNV is in the promoter region of the human Duffy and is associated with malaria (P. vivax) resistance [23]. The CC genotype of the rs2814778 are carrier of null Duffy antigen. The point mutation (rs2814778 T>C) in the promoter region of ACKR1 gene, results in a lack of expression of ACKR1 protein on RBCs. Therefore, because of the absence of Duffy antigens, malaria parasite binds the RBC, but it cannot infect them [23].

Results indicated that allele and genotype frequencies of c.125G>A (rs12075) SNP in 200 samples are indicated in (Table 3, Figure 3). The G allele was higher than the A allele in samples (72.5 and 27.5.7 %, respectively) (Figure 3). The G allele in Tabuk region and in Duba region were 71% vs 69% with no statistical significant difference (Figure4). The genotype (GG, GA and AA) frequency of the c.125 G>A (rs12075) of 10, 35, and 55 respectively (Table 4). The DARC rs12075 G>A SNV is linked with Hepatitis C virus progression [26]. Particularly cases with AG and GG genotype had reduced susceptibility to liver fibrosis and cirrhosis progression [26]. The rs12075 G>A SNP leading Glycine 42 aspartate substitution, the glycine is hydrophilic and simplest amino acid while the aspartate, a negatively charged amino acid [27, 28]. The rs12075 G>A SNV is related to variation in the serum chemokine (C-C motif) ligand 2 (CCL2) concentration and has been linked to serum interleukin-8 and RANTES concentrations [26]. Highest levels of serum CCL2 in rs12075 AA genotype perhaps because of a genotype-mediated change in Duffy function related to sulfation at Tyrosine41 rather than an alteration in expression [26]. More effective sulfation of Tyrosine 41 in the presence of the Aspartate (with negative charge) probably enhance chemokine binding to RBCs [26]. The CCL2 is involved in the recruitment of WBCs to inflammatory sites and elevated during liver fibrosis [26]. These chemokines bind and internalized by the RBCs would be released during the blood clotting [26]. The 12075 G allele exhibit decreased levels of CCL2

and CXCL8 (interleukin-8) in serum and rs12075 G allele has also been linked to chronic inflammatory diseases such as ischemia and obesity and decreased WBCs counts in Africans and European cohort [26, 29, 30]. Moreover, the SNP rs12075 was reported to interfere with metastasis potential of breast cancer through regulation of the level of the pro-malignant chemokine in the tumor microenvironment [31], and the rs12075 GG genotype can be used for the prediction of breast cancer recurrence risk[32]. Result showed that the c.265C>T, rs34599082, p.Arg89Cys are distributed in 98, 02 and 0% for the CC and CT and TT respectively (Table4). This SNP results in exchange of the Arginine, a positively charged amino acid to Cysteine, a hydrophilic and a Sulphur containing amino acid [27]. The c.265C>T, rs34599082 C>T and c.298G>A rs13962, reduce the expression of Fyb (FY*02W.01 allele, also referred to as Fyx antigen) and lead to a weak phenotype [1, 33, 34]. The TT mutation was 0% in this study (Figures5, 6). This result is consistent with the studies reported that c.265C > T (rs34599082) is less common [1, 33].

Our results showed that the allele frequencies of the c.1-67T>C (rs2814778), c.125G>A (rs12075) SNPs in Tabuk and Duba were different from the overall allele frequencies of all populations ($p<0.01$) (table 5). However, the genotype distribution of the c.265C>T, rs34599082 in Tabuk and Duba was not different from the overall allele frequencies of all populations (P – value >0.05) (Table5). To our knowledge, this is the first genotyping study of FY (ACKR1) gene in Tabuk blood donors, which may reflect the FY genotype background of this population. The limitations of the study include the limited sample size and one genotyping methodology was applied. Further large-scale studies using different genotyping techniques are required.

5. CONCLUSION

In summary we genotyped the ACKR1 gene in blood donor from Tabuk and Duba region, the ACKR1 gene was linked to Malaria infection, benign constitutional neutropenia, chronic infection, hemolytic disease of the fetus and newborn (HDFN) and acute or delayed hemolytic blood transfusion reactions. Our results shed light on the ACKR1 gene encode Duffy blood group in Tabuk northwestern Saudi Arabia further study large sample sizes with different genotyping techniques are warranted.

Author's contribution:

MAA, RM, MMJ, WMB, IFH- conceptualization, MAA, RM,E.H,R.I.B methodology; MMJ, WMB, IFH Sample Collection. MAA, RM, MMJ, and IFH: analysis and validation: MAA, RM MMJ and IFH manuscript writing. MAA, RM, MMJ, WMB, E.H,R.I.B and IFH reviewed the manuscript **.**

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Ethical Approval: This project has been ethically approved by university of Tabuk, research ethics committee, approval number: UT-125-24-2020.

Availability of data and materials: The data supporting the results of this study are available within the article.

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