

Insertion/Deletion polymorphisms do play any role in G6PD deficiency individuals in the Kingdom of the Saudi Arabia

Khalid K Alharbi, Imran Ali Khan*, Alaa Salem A Abed & Rabbani Syed

Clinical Laboratory Sciences Department, College of Applied Medical Sciences, King Saud University, P.O. Box 10219, Riyadh 11433, Kingdom of Saudi Arabia; Imran Ali Khan - Email: imranpataan@gmail.com; *Corresponding author

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Abstract:

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme in the pentose phosphate pathway (PPP) that plays an important role in protecting cells from oxidative damage by producing NADPH and reduced glutathione. G6PD deficiency is considered one of the most common genetic disorders present in the X chromosome and is the most common of enzymopathic red blood cell disorder. Angiotensin converting enzyme (ACE) plays an essential role in two physiological systems, one leading to the production of angiotensin II and the other to the degradation of bradykinin. Most studies focused on an insertion/deletion (I/D) polymorphism in intron 16 of the ACE gene as a marker for a functional polymorphism. The α_{2B} -adrenergic receptor gene (α_{2B} AR) is a three-amino acid deletion (12Glu9) polymorphism is located on chromosome 2. (Glu⁹/Glu⁹) of this polymorphism has been first time studies in G6PD individuals. We have selected 39 G6PD deficiency male individuals and PCR was carried out with the I/D polymorphisms. ACE I/D polymorphism study was carried out in G6PD individuals and showed strong association with DD genotypes and D alleles **OR=39.38, p<0.0001 (95% CI=8.80-176.1)** and **OR=38.58, p<0.0001 (95% CI=13.21-112.6)**. Another gene of α_{2B} AR I/D polymorphism study cannot show any association in DD genotype OR=0.6882, p=0.9388 (95% CI=0.2035-2.327) and with D allele OR=0.9614, p=0.9388 (95% CI=0.3482-2.653). Our study shows that G6PD deficiency is showing strong association in DD genotype and D allele of ACE gene and α_{2B} AR gene have not shown any important role and one of the reason could be the low sample size.

Keywords: G6PD, ACE, α_{2B} AR, Insertion/ Deletion, PCR and Saudi Arabia

Background:

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme in the pentose phosphate pathway (PPP) that plays an important role in protecting cells from oxidative damage by producing NADPH and reduced glutathione. In the erythrocyte, which lacks a nucleus, mitochondria and other organelles, PPP is the only biochemical pathway for generating reducing capacity [1]. Worldwide 400 G6PD deficiency individuals were asymptomatic but acute hemolysis may occur in certain conditions. It may cause chronic nonspherocytic hemolytic anemia. The common clinical manifestations of G6PD deficiency are jaundice and acute hemolytic anemia triggered

by certain drugs, infections or ingestion of fava beans [2]. G6PD deficiency is considered one of the most common genetic disorders and is the most common of enzymopathic red blood cell disorder. The disease has been reported in populations from nearly all geographical locations; however it occurs most frequently in areas where Plasmodium falciparum malaria has been endemic. Prevalence estimates are highest in Africa, Asia, the Mediterranean region and the Middle East.

The G6PD gene maps to the tip of the q arm of the X chromosome (Xq28), and consists of 13 exons encoding a 515 amino acid protein with a molecular weight of about 59 kDa [3].

Mutations in the G6PD gene may result in diminished functionality and/or stability of the G6PD enzyme, resulting in different levels of enzymatic activity and a wide range of biochemical and clinical presentations. Since it is an X-linked gene, affected hemizygous males are likely to have more severe hemolytic crises than heterozygous females who have variable proportions of G6PD normal and deficient erythrocytes. However, heterozygous females because of the mosaicism resulting from X-chromosome inactivation in somatic cells [4] may be missed by screening tests [5]. The World Health Organization (WHO) has grouped the G6PD variants into five disease classes based on enzymatic activity levels; however the clinical impact of even some of the endemic variants is not completely defined [3].

Microsatellites or short simple tandem repeats have been the genotype-based marker approach of choice for many applications where the relatedness between individuals, populations or species is sought. A microsatellite consists of a specific sequence of DNA bases or nucleotides which contains mono, di, tri, or tetra tandem repeats. Non-repetitive DNA sequence variation has been assessed through various approaches, including DNA sequencing, restriction fragment length polymorphism (RFLP) analysis, single strand conformation polymorphism (SSCP) analysis, random amplified polymorphism detection (RAPD) and amplified fragment length polymorphism (AFLP) analysis. More recently, single nucleotide polymorphisms (SNPs) are increasingly finding their application in studies of natural populations [6]. Germline variable gene codons have been deleted from the coding region as well as reports in which extra, non-template codons have been inserted into the coding region of the variable heavy and/or variable light gene segments. Such insertions and/or deletions (I/Ds) can occur by strand slippage and via homologous recombination and have been shown to occur in several human β -cell malignancies in germinal center β -cells, in human hybridomas and in peripheral blood β -cells [7].

The angiotensin-converting enzyme (ACE) gene (encoding kininase II, EC 3.4.15.1) contains a polymorphism based on the presence (insertion [I]) or absence (deletion [D]) within an intron of a 287-base-pair (Alu Sequence) nonsense DNA domain, resulting in three genotypes (DD and II homozygotes, and ID heterozygotes). ACE is an important enzyme of the renin-angiotensin-system, which is encoded by the ACE gene, located on chromosome 17q23, comprising 26 exons and 25 introns. This enzyme catalyzes the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor, and also inactivates the vasodilator bradykinin [8]. The α_{2B} AR insertion/deletion (I/D) polymorphism involves the deletion of three glutamic acids from a repeat element in the third intracellular loop of the protein. In vitro, the deletion has pronounced effects on the phosphorylation of the receptor and results in a loss of agonist-promoted desensitization.

In human studies, the α_{2B} -adrenergic receptor gene (α_{2B} AR) I/D polymorphism were found to be associated with various cardiovascular and metabolic phenotypes. A common variant (12Glu9) of the human α_{2B} AR gene encodes a receptor protein leading to the ins/del (I/D) of three consecutive glutamate residues at amino acid positions 301 to 303 has been associated with EHT, acute coronary events. In-vitro studies showed, I/D

polymorphism of α_{2B} AR gene to affect an impaired agonist-induced desensitization of the receptor [9]. In this present study we are investigating that whether I/D polymorphisms studies does play any important role in G6PD individuals in Saudi Arabian men.

Methodology:

Selection of G6PD Individuals

In this study 61 samples were included and among them 22 samples were healthy controls without any complications. This preliminary study included 39 G6PD male individuals who have visited to Central blood bank and driver school blood bank (DALLA), King Abdul-Aziz specialist Hospital in Taif city in West region of Kingdom of Saudi Arabia during the period 3rd July 2011 to 12th Aug 2011. The Age groups of these donors were 17 to 50 years with a mean age of 33.5 ± 9.96 . All the G6PD individuals were confirmed G6PD determination and quantitative test.

6ml of EDTA Blood sample was collected from every male donor and 4ml of the sample was used for hematological parameters to confirm G6PD deficiency and 2ml of the blood was used for molecular analysis. Institutional ethical committee approval and informed consent was obtained from all the subjects included in the study. The inclusion criteria of G6PD were to include male donors and female donors were excluded from the study.

DNA Isolation and Genotyping of Insertion/ Deletion Polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes using Norgen DNA extraction kit (Norgen Biotek corp, Canada). The procedure followed laid out norms for DNA extraction. The genotype analysis was performed at the Department of Genetics and clinical laboratory sciences, College of Applied Medical Sciences, King Saud University, Riyadh and Saudi Arabia.

Polymerase chain reaction (PCR) was carried out to determine the ACE and α_{2B} AR D/D, I/D, and I/I genotypes and specific Insertion/Deletion primers as shown in the **Table 1 (see supplementary material)** were chosen from Munshi A *et al* [10] and Sivenius *et al* [11] studies. Primers were synthesized by Bioserve technologies, Hyderabad, India).

A total volume of 20 μ l reaction mixture contain 2 μ l of each primer which is 100pmole, 6 μ l of sterile water and 10 μ l of 2X master mix which includes MgCl₂, 10x Taq buffer, 10 unit of Taq DNA polymerase (Norgen Biotek corp, Canada) and the 2 μ l template DNA was used for amplification of I/D polymorphisms of ACE gene and α_{2B} AR gene.

For the genotyping I/D genotype variant of the ACE gene was determined by PCR amplification. PCR products of 190bp (without insertion) and 490bp (with 287-bp Alu insertion) were detected by electrophoresis on agarose gel containing ethidium bromide. Amplification of the PCR products, which is a 490-bp fragment for the I allele and a 190-bp fragment for the D allele, was carried out using the following: The PCR profile consisted of 35 cycles (denaturized at 94°C in 5 minute, annealing at 59°C in 30 seconds and extension at 72°C for 5 minute) was used.

The PCR cycling conditions were carried out on Thermal Cycler (Applied Biosystems, Hercules, California, USA). The 1st Exon of the polymorphic $\alpha_2\text{B}$ AR gene the initial denaturation was set up for 5 minutes at 95°C followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 57°C, extension for 45 seconds at 72°C and the final extension was at 72°C for 7 minutes. The insertion band is considered as 112bp which represents the I allele and 103bp is identified as deletion band which represented the D allele. Insertion/Deletion of the amplified PCR products were clearly separated on 2.5% agarose gel (Cambrex, East Rutherford, NJ, USA) which has been performed in Origins electrophoresis (Elchrom Scientific AG, Cham, Switzerland).

Statistical Analysis

All the statistical analysis was carried out by using SPSS (Chicago, IL, USA) software version 19.0 for Microsoft Windows®. Clinical characteristics of all the subjects were expressed as mean \pm SD. Continuous variables were compared between the groups by using two-tailed student's *t* test. Allelic frequencies were calculated by gene-counting method and the genotypic and allelic distribution of I/D polymorphism of $\alpha_2\text{B}$ AR gene were analyzed using chi-square test. The effect of I/D genotypes of $\alpha_2\text{B}$ AR was analyzed using general linear model ANOVA for clinical characteristics. A level of *P* <0.05 was considered statistically significant.

Results & Discussion:

I/D polymorphisms were identified based on the band sizes of ACE and $\alpha_2\text{B}$ AR genes. Over all 39 men were participated were in this study and women's were excluded from this study. G6PD individuals were in the age range of 17-50 years with a mean age of 33.5 \pm 9.96. It was seen that G6PD individual's blood groups were A, B and O of both positive and negative blood groups and none of the G6PD individual were not having both AB positive and negative blood groups. **Table 2 (see supplementary material)** shows the individuals participated in the study with their blood groups.

ACE I/D polymorphism study was carried out in G6PD individuals in Saudi Arabian men. The frequencies of ACE DD, ID and II genotypes among G6PD patients were 82%, 7.7% and 10.2% respectively. The percentage of D allele was 85.9% and of the I allele was 14.1%. In the control subjects, the distribution of ACE DD, ID and II genotypes was 9%, 9% and 81.9% respectively. The allele frequencies was 13.6% and 86.3% for the D and I alleles, respectively. When we have compared the ACE DD genotypes and D allele with the cases and controls we have found a strong association **OR=39.38, p<0.0001 (95% CI=8.80-176.1)** and **OR=38.58, p<0.0001 (95% CI=13.21-112.6)**. Similarly, the difference of D allele frequencies between patients and controls was found to be strong significant; 82% for patients and 9% for controls. **Table 3 (see supplementary material)** is showing the genotypes and allele frequencies of 2 different genes.

Another gene of $\alpha_2\text{B}$ AR I/D polymorphism study was carried out with G6PD individuals in Saudi population. In the G6PD group 4/39 (10.2%) cases showed DD genotype, 04/39 (10.2%) showed ID and 31/39 (79.5%) showed DD genotype. Among the cases 12 (15.4%) showed D allele and 66 (84.6%) showed I allele. In the control group 01/22 (4.5%) showed DD genotype,

05/22 (22.7%) showed ID genotype and 16/22 (72.7%) showed II genotype. In the control group 07 (15.4%) showed D allele and 37 (84.1%) showed I allele. A chi square test between the cases and control of D allele did not show a significance difference OR=0.9614, p=0.9388 (95% CI=0.3482-2.653) D Allele. None of the genotypes or allele did not show any association in $\alpha_2\text{B}$ AR I/D polymorphism in Saudi population OR=0.6882, p=0.9388 (95% CI=0.2035-2.327) DD genotype **Table 4 (see supplementary material)**.

This study is a comprehensive investigation conducted in 2011 to determine the frequency of G6PD deficiency and its variants in Saudi men. For the G6PD deficiency there are many hematological and genetic studies were conducted in the kingdom of Saudi Arabia [12]. In the G6PD gene 13 exons were present and complete genetic sequencing analysis were conducted in the Arab population. Till now there are no studies were conducted on other gene polymorphisms apart from G6PD gene. In our study we have conducted I/D gene polymorphism studies to rule out whether I/D polymorphisms play any role in G6PD individuals. We have selected 2 genes i.e. ACE and $\alpha_2\text{B}$ AR genes. The importance of ACE gene is a zinc metalloprotease widely distributed on the surface of epithelial and endothelial cells. ACE gene consists of 26 exons and spans 21 kb on human chromosome 17. The sequence codes a 1306 amino-acid protein including a signal peptide. The gene product of ACE is composed of two homologous domains with two active sites. It has a common polymorphism which consists of the presence (I allele) or absence (D allele) of a 287 bp Alu repeat sequence within intron 16. ACE activity in individuals with DD genotype was found to be more than 8 times in those with II genotype. Subjects with ID genotype exhibit intermediate levels of ACE [13].

$\alpha_2\text{B}$ AR I/D polymorphism is leading to a detection of three glutamic acids from a glutamic acid repeat element (Glu X 12, amino acids 297-309), located at the third intracellular loop of the receptor protein has been identified. The important selection of this polymorphism was found to be more associated with reduced basal metabolic rate in obese individuals. In this study most of the men over weight and obese. From this gene study we didn't find any association either with allele or with genotypes. The reason that the $\alpha_2\text{B}$ AR I/D polymorphism could not show any significant effect in G6PD individuals could be due to a much strong impact of G6PD deficiency as per compared with that of this gene variant.

There are multiple studies were carried out in G6PD individuals and have been reported. The African A mutation studies and the two mutations i.e. C563T and 1311 C>T G6PD Mediterranean and G6PD Sibri with different polymorphic rates reinstate earlier studies. The G6PD Mediterranean, having C→T transition at nucleotide 563 of exon 6 with most prevalent allele has been reported from Mediterranean Middle East and India as well [2]. This group has been carried out the genetic work on the complete exon sequence of 6 and 7 in the G6PD gene in Jeddah. G6PD gene polymorphism study was carried out in G6PD deficient in more than 20 Arab countries and none of them has been worked on I/D polymorphisms. Till date there are no I/D polymorphism studies in G6PD patients and this will be the first genetic study in G6PD individuals from the Saudi Arabia.

In our study we have found that ACE I/D polymorphism study was found to be strongly associated and 82% of individuals were found to be deletion of Alu sequence, which means prone for the disease and in the $\alpha_2\text{B}$ AR gene I/D polymorphism didn't show any association with the disease. We have mentioned one of the reasons earlier and the other reason could be because of lack of sample size. In our study we have chosen only 39 samples because all the men participated in this study is purely Saudis from Taif city. We recommend having a large sample size can help to rule out the disease whether it is showing any important role or not.

From our study we conclude that G6PD deficiency is showing strong association in DD genotype and D allele of ACE gene. Any individual effecting DD genotype and D allele is prone for the disease in ACE gene. We could not able to show any association in $\alpha_2\text{B}$ AR gene I/D polymorphism. Large sample size has been recommended to study this disease.

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Supplementary material:

Table 1: Genotype distribution of the polymorphisms examined in this study

GENE	SNP	rs no	FORWARD PRIMER	REVERSE PRIMER	FRAGMENT	ANNEALING TEMP
ACE	INTRON 16	rs4646994	CTGGAGACCACTC CCATCCTTTCT	GATGTGGCCATCA CATTTCGTCACGAT	490bp	59°C
α_2B AR	EXON 1	rs4426564	AGGGTGTTTGTGG GGCATCT	CAAGCTGAGGCCG GAGACT	112bp	57°C

Table 2: G6PD Individual and percentages of their blood groups

Blood Group	Positive	Negative
A	10 (25.65%)	01 (2.56%)
B	04 (10.25%)	01 (2.56%)
AB	00 (0%)	00 (0%)
O	22 (56.41%)	01 (2.56%)

Table 3: Genotype and allele frequency of ACE and α_2B AR

Genotypes and Alleles	ACE Cases (n=39)	ACE Controls (n=22)	α_2B AR Cases (n=39)	α_2B AR Controls (n=22)
II	04 (10.2)	18 (81.9)	31 (79.5)	16 (72.7)
ID	03 (7.7)	02 (9)	04 (10.2)	05 (22.7)
DD	32 (82)	02 (9)	04 (10.2)	01 (4.5)
I	11 (14.1)	38 (86.3)	66 (84.6)	37 (84.1)
D	67 (85.9)	06 (13.7)	12 (15.4)	07 (15.9)

Table 4: Statistical analysis for G6PD individuals

S.No	Genotypes	ACE	α_2B AR
1	DD Vs ID+II	OR-45.71;95%CI=8.62-242.3; p<0.0001	OR-2.4;95%CI=0.2512-22.93; p=0.4349
2	ID+DD Vs II	OR-39.38;95%CI=8.80-176.1; p<0.0001	OR-0.6882;95%CI=0.2035-2.327; p=0.5466
3	ID Vs II+DD	OR-0.833;95%CI=0.12-5.411; p=0.84	OR-0.3886;95%CI=0.0092-1.635; p=0.1875
4	D Vs I	OR-38.58;95%CI=-13.21-112.6; p<0.0001	OR-0.9614;95%CI=0.3482-2.653; p=0.9388