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Thrombophilic gene polymorphism studies in G6PD deficient individuals from Saudi population

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Abstract:
We performed a study to evaluate the role of three single nucleotide polymorphisms (SNPs), factor V Leiden G1691A (FVL), prothrombin gene mutation G20210A (PRT or FII-G20210A) and methylenotetrahydrofolate reductase variant C677T (MTHFR-C677T), as risk factors for G6PD in Saudi populations. Our results did not show any association with the three Thrombophilic genes with FVL gene, no statistical analysis have shown any association with either allele or genotype frequencies OR=0.566, p=.0.667, (95% CI=0.014-22.48) and OR=0.569, p=0.251¸ (95% CI=0.014-22.96).In PRT gene G20210A for G Vs A, p=0.774; OR=0.566 (95%CI; 0.011-29.6); AA+GA Vs GG; p=0.502; OR=0.569 (95%CI=0.010-2969). G and A allele frequencies were similar between cases and controls with no statistical significance. In the MTHFR gene none of the genotypes or allele frequency cannot show any association OR=1.281, p=.0.667, (95% CI=0.414-3.958) and OR=1.1.172, p=0.800¸ (95% CI=0.343-4.008). Similarly, the difference of T allele frequencies between patients and controls was not found any association. In conclusion, our finding indicates that the prevalence of G1691A, G20210A and C677T mutations in G6PD deficient individuals is not statistically different compared to normal subjects and G6PD is not associated with these thrombophilic mutations in Saudi population.

Keywords: G6PD, FVL, PRT, MTHFR, PCR and Saudi Population

Background:
Glucose-6-phosphate dehydrogenase (G6PD) enzyme is critically important for protecting erythrocytes from oxidative stress and intravascular hemolysis. G6PD is an X-linked gene encoding the first enzyme of the pentose phosphate pathway (PPP), a NADPH-producing dehydrogenase. Because erythrocytes do not generate NADPH in any other way than PPP, they are susceptible to oxidative damages. G6PD deficiency is a prime example of a hemolytic anemia due to an interaction between an intra-corpuscular and an extra corpuscular cause. In the majority of cases, an exogenous agent, such as an exposure to oxidant stress triggers hemolysis **[1].** G6PD gene is located at the telomeric region of the X chromosome (band Xq28), consisting of 13 exons and 12 introns. It encodes 515 amino acids and a GC-rich (more than 70%) promoter region. G6PD-deficient erythrocytes are more susceptible to destruction by oxidative stress than normal erythrocytes due to the lower NADPH levels **[2].** Individuals with this genetic defect may exhibit non-immune hemolytic anemia in response to a number of stimuli, most commonly infections or exposure to certain medications or chemicals **[3].** G6PD is X-linked, and so deficient variants are expressed more commonly in males than in females. Worldwide, an estimated 400 million people are G6PD deficient with the distribution corresponding to areas in which malaria is, or has been, prevalent. There is some evidence that hemizygous males may be protected against severe malaria **[4].**

In 1994, Bertina et al described a common variation (G1691A) in the Factor V Leiden (FVL) gene as a molecular defect responsible for activated protein C (APC) resistance, a previously unrecognized mechanism of inherited thrombophilia. FVL gene variant is caused by a single point mutation at nt1691, leading to the replacement of arginine at

position 506 in the APC cleavage site of factor Va by glutamine. This mutation represents one of the most important risk factors for inherited thrombophilia (Mozafari H *et al.,* 2009). A nucleotide transition at G20210A in the 3'untranslated region of the prothrombin (PRT or FII) gene was associated with nearly a 25% increase in plasma thrombin activity and a 2.7-fold increased risk for venous thrombosis. G20210A is associated with increased plasma levels of PRT. It is the 2nd most common inherited risk factor for thrombosis **[5, 6].** The FVL (G1691A) and PRT (G20210A) mutations are 2 such examples; each has been found to be associated strongly with spontaneous and recurrent venous thromboembolism. Similarly, a thermolabile variant of methylenetetrahydrofolate reductase (MTHFR) is an enzyme involved in the folate-dependent metabolism of homocysteine, increases the risk for deep vein thrombosis and pulmonary embolism. MTHFR is a key enzyme in folate and homocysteine (Hcy) metabolism. A single point mutation causing a C-T substitution at nucleotide 677 and from the valine to alanine in the enzyme of the MTHFR gene has been associated with a thermolabile enzyme form of low biological activity. C677T MTHFR polymorphism is associated with low folate and vitamin B12 levels. The role of high total Hcy serum or plasma levels received considerable interest after these factors were implicated as an important risk factor for vascular and several other diseases. The three single nucleotide polymorphisms (SNPs) are most recognized as predisposing to thrombosis are FVL mutation (G1691A), PRT mutation (G20210A)) and MTHFR variant (C677T).

Although studies performed on epidemiology, genetics and molecular biology have broadened the information on G6PD deficiency; there are still no reliable and validated methods to test drug hemolytic potential in G6PD deficient patients **[7].** In our study we have confirmed the G6PD individuals by G6PD determination test which is a hematological test and later on we have performed molecular test with PCR-RFLP followed by 2% electrophoresis. We have chosen to screen for the FVL (G1691A), PRT (G20210A) and MTHFR (C677T) mutations. The FVL and the PRT gene mutations are associated with increased risk of venous thromboembolism and possibly myocardial infarction and stroke. The MTHFR mutation increases plasma homocysteine, which has emerged as a potential risk factor for cardiovascular diseases. We aimed to assess the role of these SNPs (i.e. Thrombophilic mutations and MTHFR C677T) as they play any risk factors for G6PD deficient individuals among Saudi population.

Methodology:

A total of 61 patients among which G6PD deficient individuals was 39 and were recruited from different institutes like Central blood bank, Driver school blood bank (DALLA) and King Abdul-Aziz specialist Hospital in Taif city in West region of Kingdom of Saudi Arabia during the period of July 2011 to Aug 2011. They were compared with 22 normal subjects without any complications and 6ml samples was collected in EDTA anticoagulated tubes and 4ml of the sample was used for hematological test to confirm the G6PD deficiency for G6PD determination test and G6PD deficiency using fluorescent spot test (Boehringer Mannheim GmGH, West Germany). 2ml of the EDTA sample was used for the identifying the molecular analysis. Institutional ethical committee approval and informed consent was obtained from all the subjects included in the study. The inclusion criteria of G6PD deficiency were to include male donors. All types of G6PD were excluded using a questionnaire that was female. Fluorescence is produced due to reduction of NADP to NADPH. This reaction is coupled with oxidation of glucose-6-phosphate to 6-phosphogluconate and catalyzed by G6PD. Specimens with G6PD activity of less than 20% of normal do not fluoresce, as the small amount of NADPH formed is re-oxidized by the glutathione present in the reagents**.**

Genomic DNA was isolated from peripheral blood samples according to a standard protocol described elsewhere (Norgen, Germany). DNA was dissolved in TE Buffer (~100ng/µl DNA concentration) and stored at-80ºC until further process. The genotype analysis was performed at the Department of 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 Thrombophilia genotyping of 3 genes (FVL-PTR-MTHFR) was performed by using PCR-RFLP and specific primers as shown in the **Table 1 (see supplementary material)** were chosen from the reference **[5]** studies. Primers were synthesized by Bioserve technologies, Hyderabad, India).

Statistical Analysis

Clinical characteristics of all the subjects were expressed as mean± SD. Statistical analysis was performed by using SPSS program. Allele frequencies were calculated by gene counting method. *P* values<0.05 were considered significant, <0.01 highly significant and <0.0001 extremely significant. Odds ratios were also calculated for the samples.

Result & Discussion:

In this study 39 G6PD deficiency male individuals were included. The age range was started from 17-50 years with a mean age of 33.5+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 having both AB positive and negative blood groups. **Table 2** (**see supplementary material)** shows the individuals participated in the study with their blood groups.

G1691A allele and genotype frequencies

To genotype G1691A the 241bp PCR product was obtained, which on digesting with HindIII restriction enzyme give fragments of 241bp indicating GG, 241/209/32bp indicating GA and 209/32bp indicating AA genotype. In the G6PD deficiency group, the GG (100%) and none of the genotype (GA and AA) were present. In the control group GG genotype was found to be 95% and GA was found to be 5%. No statistical analysis have shown any association with either allele or genotype frequencies OR=0.566, p=.0.667, (95% CI=0.014-22.48) and OR=0.569, p=0.251¸ (95% CI=0.014-22.96).

G20210A allele and genotype frequencies

A 345bp wild type PCR product of G20210A was digested with HindIII restriction enzyme to generate a fragment of 322bp which is a mutant type and heterozygotes are expected to have both the fragments. Complete GG (100%) genotypes were present in both the cases and control. GA and AA were completely absent in all the samples. No significant evidence of association was observed for any of the phenotypes investigated with G6PD cases. For G Vs A, p=0.774; OR=0.566 (95%CI; 0.011-29.6); AA+GA Vs GG; p=0.502; OR=0.569

(95%CI=0.010-2969). G and A allele frequencies were similar between cases and controls with no statistical significance.

C677T allele and genotype frequencies

The frequency of MTHFR C677T mutant allele (T) was highest in our study when comparing with G1691A and G20210A mutations. The C677T PCR product was obtained to be 198bp, later on digestion with HinfI restriction enzyme give the fragments of 198bp indicating CC, 198/175/23bp indicating CT and 175/23bp indicating TT genotype. The frequencies of C677T CC, CT and TT genotypes among G6PD deficiency males were 74.4%, 23% and 2.56% respectively. The percentage of T allele was 14.1% and C allele was 85.9%. G6PD cases were compared with the normal subjects and the distribution of CC, CT and TT genotypes was 77.3%, 22.7% and 0%. TT genotype was absent in the control groups. The allele frequency of control subjects of C and T allele was 88.6% and 11.4%. We have compared the C677T cases with the controls. None of the genotypes or allele frequency cannot show any association OR=1.281, p=.0.667, (95% CI=0.414-3.958) and OR=1.1.172, p=0.800, (95% CI=0.343-4.008). Similarly, the difference of T allele frequencies between patients and controls was not found any association. **Table 3 (see supplementary material)** shows the Genotypes and Allele frequencies of FVL, PRT and MTHFR genes involved in this study.

The G6PD deficiency is one of the commonly occurring genetic enzyme disorders known to affect millions of individuals worldwide and an increased risk for venous thrombosis. It is linked to the Mediterranean basin. In G6PD deficiency males may be either G6PD normal or deficient hemizygotes, whereas females may be normal homozygotes, deficient homozygotes, or heterozygotes. In the G6PD gene 13 exons were present and complete genetic sequencing analysis were conducted in the Saudi Arabia individuals. Till now there are no studies were conducted on other gene polymorphisms apart from G6PD gene. In our study we have conducted thrombophilic gene mutational studies to rule out whether G1691A, G20210A and C677T mutations may play any role in G6PD individuals.

FVL is a point mutation which impairs the proteolytic degradation of the factor V by protein C that makes it active for a long time. This abnormality of the hemostasis system is associated with an increased risk of venous thrombosis. The prevalence of the mutation varies among different populations, being high in Europeans and almost absent in the populations of Southeast Asia, Polynesia and Africa. The largest population study among Europeans revealed a mean frequency of 2.78% with a peak value of 12% in Cyprus with decreasing frequency from south to north and from west to east. 11% of the prevalence in G6PD deficiency was found in the Dalmatian population. In an Iran the prevalence rate was 2.5% and in our population this mutation was completely absent in the G6PD deficiency **[8].**

Prothrombin, the precursor of thrombin is a vitamin K-dependent glycoprotein that plays a central role in blood coagulation. Activated by factor Xa, thrombin converts fibrinogen to fibrin. The prothrombin (PRT or FII) gene is located on chromosome 11 near the centromere, spans approximately 21 kb, and is composed of 14 exons and 13 introns, PRT G20210A represents a gain-of-function mutation which affects the 3'-terminal nucleotide of the 3' untranslated region (UTR) of the mRNA. PRT is the second most common cause of familial thrombophilia is a mutation in the 3' untranslated region of the PRT gene (G20210A). This mutation leads to an elevated level of prothrombin because of increased synthesis but does not lead to an altered aminoacid sequence or altered function **[9].** Elevated levels of prothrombin occur due to increased cleavage site recognition, increased 3' end processing and increased mRNA accumulation and protein synthesis. Resistance to activated protein C secondary to hyper prothrombinemia has also been reported.

The prevalence of this gene variant varies widely worldwide, being virtually absent in Africa, Asia, and natives of America, while being common in Europe. In the general European population the prevalence has been reported to range from 0.7% to 4%, and 5.5% in the UK. In contrast, PRT or factor II G20210A was found in only one of 441 African Americans and was absent among Japanese subjects. In unselected patients with venous thrombosis, the prevalence ranges from 4% to 8%. Abu Amero *et al* 2002 studies have shown that 1.7% prevalence rate for G20210A mutation in the Saudi Arabia. Their sample size of overall study was 1206 which is almost all 20 times more than our samples and this were not from G6PD deficiencies. In our study heterozygotes and mutants were completely absent and it indicates that the person who had can be prone for the disease. G20210A mutation had no role in the G6PD deficiency.

MTHFR gene is having a genetic factor to contribute in the thrombotic manifestations of inflammatory bowel disease. MTHFR is involved in the one-carbon cycle, which is of importance for nucleotide synthesis and methylation of DNA, membranes, proteins and lipids. Most common MTHFR gene polymorphisms are C677T and A1298C. Mutations in this gene result in a decrease of the enzyme activity that leads to mild hyperhomocysteinemia. A study from Szczeklik A *et al* suggested mutations in this gene for allele C677T and A1298C to be a tendency to associate with coronary artery disease (CAD), in both homozygous and heterozygous carriers even when blood homocysteine levels were not elevated **[10 & 11].**

The frequency of MTHFR gene mutations varies between different population and also among racial and ethnic groups. In our study group the frequency was found to be 2.5%. In the Europeans the frequency of mutant T allele is 18.6%, Asian Population is 20.8%, 32.2% in an American population, 6.6% in an African population, 4.7% in an Australasian population and 29.1% in the Iran population **[12].** The association between this variant and venous thromboembolism/CAD has been controversial. The frequency of T allele in our healthy population was found to be (0 %) when observed in European (18.6%) or the Asian population (20.8%) and lower than in the American population (32.2%). Higher prevalence of mutant T allele (29.1%) existed in G6PD deficient individuals in an Iran compared to healthy population, but was not statistically significant when compared with our study.

Our results indicate that none of these prothrombotic gene mutations are important risk factors for G6PD deficiency in the Saudi population. It indicates that these mutations cannot be used to identify patients with hematological disorders and our results are not consistent with previous findings in the Mozafari H *et al* studies from the western region of the Iran in an Arab population. We cannot completely rule out the possibility of an

association in an Arab population. First, although this is a relatively small study of these mutations in this G6PD deficient male population, the sample size is still limited and the confidence intervals relatively wide. Secondly, confounding from other prognostic variables may have played a role. To our knowledge only one study from Iran has studied G6PD deficiencies with thrombophilic genes and this study also does not investigated prothrombin gene mutations are risk factors for G6PD deficiency. We cannot find any significant trend toward a higher risk of G6PD deficiency among patients who were carriers of the prothrombin gene mutations selected in this study.

Conclusion:

In conclusion, our finding indicates that the prevalence of G1691A, G20210A and C677T mutations in G6PD deficient individuals is not statistically different compared to normal subjects and G6PD is not associated with these thrombophilic mutations in Saudi Arabia also. These could be due to the small sample size. Our study suggests that large sample may be required to rule out the disease.

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Supplementary material:

Table 1: Different types of SNPs were examined in this study.

Gene	Mutation	rs no	Forward Primer	Reverse primer	Fragment	Annealing Temp	Enzyme
FVL	G1691A	rs6020	TCAGGCAGGAAC AACACCAT	GGTTACTTCAAGGA CAAAATACCTGTAA AGCT	241bp	56°C	HindIII
PRT (FII)	G20210A	rs1799963	TCTAGAAACAGT TGCCTGGC	ATAGCACTGGGAGC ATGAAGCAAGC	345bp	60°C	HindIII
MTHFR	C677T	rs1801133	TGAAGGAGAAG GTGTCTGAGGGA	AGGACGGTGCGGTG AGAGTG	198bp	68°C	HinfI

Table 2: G6PD Individual and percentages of their blood groups

Blood Group	Positive	Blood Group
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 frequencies of FVL, PRT and MTHFR genes.

Genotypes and Alleles	FACTOR V Cases (n=39)	FACTOR V Controls (n=22)	PROTHROMBIN Cases (n=39)	PROTHROMBIN Controls (n=22)	MTHFR Cases (n=39)	MTHFR Controls (n=22)
Normal	39 (100)	21 (95)	39 (100)	22 (100)	29 (74.4)	17 (77.3)
Heterozygous	00 (0)	01 (5)	00 (0)	00 (0)	09 (23)	05 (22.7)
Variant	00 (0)	00 (0)	00 (0)	00 (0)	01 (2.56)	00 (0)
Wild Type	78 (100)	43 (97.8)	78 (100)	44 (100)	67 (85.9)	39 (88.6)
Mutant Type	00 (0)	01 (2.2)	00 (0)	00 (0)	11 (14.1)	05 (11.4)